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ATIVIDADE BIOLÓGICA DE RESÍDUOS DE VINIFICAÇÃO DE *Vitis labrusca*

GUSTAVO SCOLA

CAXIAS DO SUL

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Tese de doutorado apresentada ao Programa de Pós-Graduação em Biotecnologia da Universidade de Caxias do Sul, como parte dos requisitos necessários à obtenção do grau de Doutor em Biotecnologia. Orientadora: Profª. Drª. Mirian Salvador.

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AIF	=	Fator de indução de apoptose
ATP	=	Trifosfato de adenosina
CAT	=	Catalase
DNA	=	Ácido desoxirribonucleico
Her-2	=	Receptor do fator de crescimento epidérmico 2
NDUFS1	=	NADH desidrogenase (ubiquinona) Fe-S proteína 1 - (NADH-coenzima Q redutase)
NDUFS7	=	NADH desidrogenase (ubiquinona) Fe-S proteína 7 - (NADH-coenzima Q redutase)
NDUFS8	=	NADH desidrogenase (ubiquinona) Fe-S proteína 8 - (NADH-coenzima Q redutase)
NDUFV1	=	NADH desidrogenase (ubiquinona) flavoproteína 1
NDUFV2	=	NADH desidrogenase (ubiquinona) flavoproteína 2
PARP	=	Poli ADP ribose polimerase
PTZ	=	Pentilenotetrazol
SNC	=	Sistema nervoso central
SOD	=	Superóxido dismutase
TNF	=	Fator de necrose tumoral
VLE	=	Extrato de *Vitis labrusca*

RESUMO

RESUMO

Anualmente, a produção mundial de vinhos gera cerca de 20 milhões de toneladas de resíduos, os quais, na maior parte, são descartados no meio ambiente. Dados prévios demostraram que é possível obter-se compostos flavan-3-ol a partir de diferentes resíduos de vinificação, com significativa atividade antioxidante. Em vista disso, o objetivo deste trabalho foi investigar as atividades neuroprotetora, hepatoprotetora, genotóxica, mutagênica e citotóxica do extrato aquoso de sementes de resíduos de vinificação de *Vitis labrusca*, variedade Bordo, avaliando o mecanismo de ação do mesmo. Os resultados mostraram que o extrato minimiza os danos oxidativos aos lipídeos e proteínas em córtex cerebral, cerebelo, hipocampo, e no fígado de ratos tratados com pentilenotetrazol. Além disso, o extrato não alterou o comportamento locomotor dos ratos e não apresentou efeito genotóxico (ensaio cometa) ou mutagênico em linfócitos de ratos e células de levedura *Saccharomyces cerevisiae*, respectivamente. O tratamento com o extrato diminui a depleção das proteínas do complexo I da cadeia transportadora de elétrons na mitocôndria em células de neuroblastoma humano (SH-SY5Y) tratadas com H_2O_2. Em doses de 0,1 e 1mg/mL, o extrato mostrou-se citotóxico para as linhagens de câncer de fígado (HepG2) e de mama (MCF-7). Este efeito foi acompanhado pelo aumento da produção de óxido nítrico, da expressão de p53 e da fragmentação do DNA. Uma diminuição significativa nos níveis de expressão da PARP total foi observada na linhagem HepG2. Na linhagem MCF-7, o tratamento com o extrato de *V. labrusca* aumentou a expressão das proteínas Bax e AIF, e diminuiu a expressão da PARP total. A expressão da proteína Her-2 apresentou-se diminuída em células HepG2 e MCF-7

tratadas com o extrato. Estes resultados mostram importantes efeitos biológicos do extrato de resíduos de vinificação de *V. labrusca*, variedade Bordo.

ABSTRACT

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Every year, the worldwide wine production generates about 20 million tons of waste, which usually end up being discarded in the environment. Previous data showed that it is possible to obtain flavan-3-ol compounds from different winery wastes with significant antioxidant activity. Thus, the aim of this work was to investigate the neuroprotective, hepatoprotective, genotoxic, mutagenic and cytotoxic activities of aqueous winery seeds extract from *Vitis labrusca*, *cv*. Bordo. The results showed that the extract minimizes the oxidative damage to lipids and proteins in the cerebral cortex, cerebellum, hippocampus and liver of rats. Also, the extract did not alter locomotor behavior, and it was non-genotoxic (comet assay) or non-mutagenic in lymphocytes from rats, and in *Saccharomyces cerevisiae* cells, respectively. The treatment with the extract was able to prevent depletion of mitochondrial eletron transfer chain complex I proteins in neuroblastoma cells (SH-SY5Y) treated with H_2O_2. At doses of 0.1 and 1mg/mL, the extract was found to be cytotoxic to liver (HepG2) and breast (MCF-7) cancer cell lines. This effect was accompanied with an increase in nitric oxide production, increase in the expression of p53, and increase in DNA damage index. A significant decrease in the expression levels of total PARP was observed in HepG2 cell line. In MCF-7 cells, *V. labrusca* extract treatment increased the expression levels of Bax and AIF proteins, and decreased the expression levels of total PARP. The expression levels of Her-2 protein were found to be decreased in HepG2 and MCF-7 cells after *V. labrusca* extract treatment. These results show significant biological effects of *V. labrusca* winery wastes extract, *cv*. Bordo.

INTRODUÇÃO

1. INTRODUÇÃO

1.1 Vitis e compostos fenólicos

As videiras classificam-se no grupo das Cormófitas, divisão Spermatophyta, subdivisão Angyosperma, classe Dicotyledoneae, ordem Rhamnales, filo Terebintales-Rubiales, família Vitaceae ou Ampelidaceae. Dentre os gêneros pertencentes a esta família destaca-se o gênero *Vitis*, pois apresenta grande importância econômica, social e histórica, sendo as espécies labrusca e vinifera as mais cultivadas no mundo (Camargo, 1994, Giovannini, 1999).

O gênero *Vitis* sp foi introduzido no Brasil em 1532, com a espécie V*itis vinifera*, originária de Portugal (Camargo, 1994). Entre os anos de 1830 a 1850, foram trazidas cultivares de origem americana, denominadas *Vitis labrusca.* Esta espécie ocorre desde o sudeste do Canadá até a costa leste dos Estados Unidos, sendo a espécie americana há mais tempo conhecida. Ambas as espécies de videiras difundiram-se rapidamente em todo país (Camargo, 1994, Giovannini, 1999) e, a partir da segunda metade do século XIX, a vitivinicultura passou a ter importância comercial, principalmente, para os estados do Rio Grande do Sul, Santa Catarina, São Paulo, Minas Gerais, Bahia e Pernambuco.

A espécie *V. vinifera* é a mais utilizada para a elaboração de vinhos finos (Winkler *et al.*, 1997), principalmente a partir das cultivares Cabernet Sauvignon e Merlot (Giovannini, 1999). A *V. labrusca* é a espécie mais empregada para a produção de vinhos comuns, pois engloba variedades mais produtivas e, portanto, com custo de produção inferior ao da vinifera. Entre as principais cultivares americanas estão a Bordo, também conhecida como

Yves e a Isabel, também chamada de Isabella (Camargo, 1994, Winkler *et al.*, 1997, Giovannini, 1999).

Os dados mais recentes mostram que, em 2011, o Brasil produziu 1.463.481 toneladas de uva, sendo que, aproximadamente, 40% foi destinada à elaboração de vinhos (Ribeiro de Mello, 2012). A produção de *V. labrusca,* em 2009, foi de 498.383,917 t, sendo 76.907,674 t da variedade Bordo (Embrapa, 2010). Apenas no Estado do Rio Grande do Sul, foram produzidos 257.840.749 litros de vinho de mesa, representando mais da metade da produção nacional (Ribeiro de Mello, 2012).

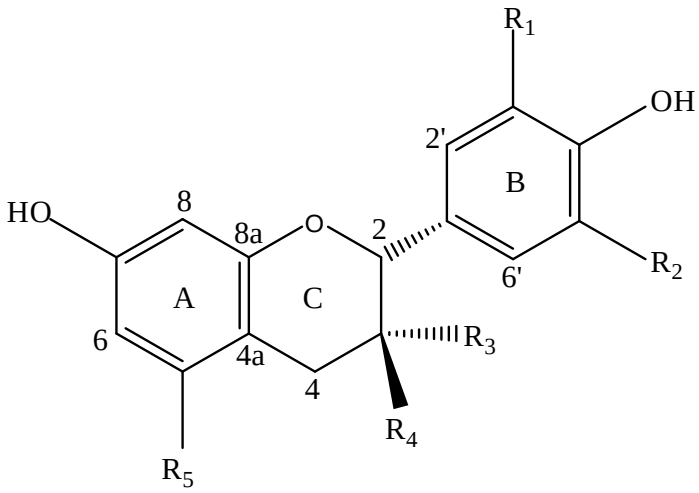
Como resultado do processo de vinificação, é gerada uma grande quantidade de resíduos (cerca de 13% do peso da uva processada), que inclui cascas, galhos e sementes, sendo as sementes as mais abundantes (em cada 100 kg de resíduo úmido gerado pelas indústrias existe cerca de 10 a 12 kg de sementes)(Oliveira *et al.*, 2003). A maior parte destes resíduos são descartados no meio ambiente, e em menor escala, utilizados como adubo (Torres *et al.*, 2002) ou mais recentemente, nas caldeiras das empresas vinícolas.

O gênero *Vitis* é rico em compostos antioxidantes, principalmente polifenóis, sendo os mais abundantes os flavonóides, os quais apresentam uma estrutura hidrocarbonada do tipo C_6-C_3-C_6 (difenilpropano), originada do ácido chiquímico e de três derivados de acetato (Aron and Kennedy, 2008). Estes compostos são divididos em diferentes classes (flavan-3-ol, flavona, flavonol, flavanona, flavononol, antocianidina, chalcona, aurona)(Monagas *et al.*, 2003, Ashraf-Khorassani and Taylor, 2004, Kammerer *et al.*, 2004, Koyama *et al.*, 2007, Saiko *et al.*, 2008). As sementes da uva são ricas em compostos flavan-3-ol (Fuleki and Ricardo-Da-Silva, 2003), principalmente, (+)-catequina, (-)-epicatequina, (-)-epicatequina 3-*O*-galato, oligômeros e polímeros de flavan-3-ols e o ácido gálico (Gabetta *et al.*, 2000, Ashraf-Khorassani and Taylor, 2004, Kammerer *et al.*, 2004, Koyama *et al.*, 2007, Scola *et al.*, 2010).

Na casca são encontrados os flavonóis (quempferol, quercetina e miricetina), as

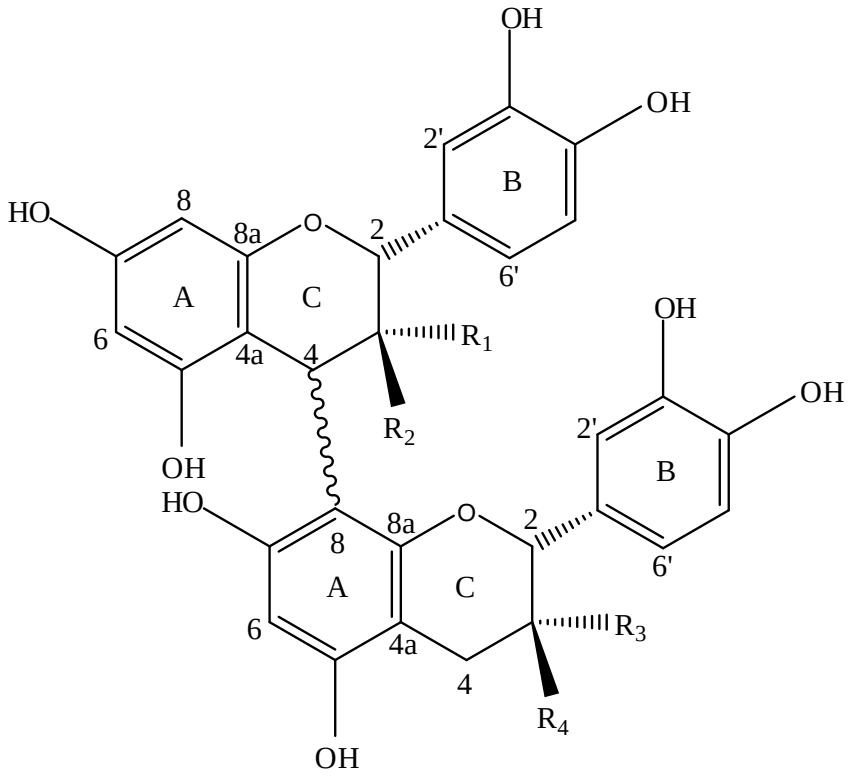
antocianinas (cianidina, delfinidina, peonidina, petunidina, malvidina), os estilbenos (resveratrol) e os ácidos fenólicos (ácido cafeiltartárico e o ácido pcumariltartárico)(Koyama *et al.*, 2007, Saiko *et al.*, 2008). A polpa da uva é rica em compostos não flavonóides, como o ácido ferrúlico, ácido p-cumárico e o ácido cafeico (Di Stefano, 1996).

Os compostos flavan-3-ol também recebem a nomenclatura de taninos. Neste grupo estão presentes, além dos monômeros, os dímeros de flavan-3-ols, como as protoantocianidinas B-1 ((–)-epicatequina-(4β→8)-(+)-catequina), B-2 ((-)-epicatequina-(4β→8)-(–)-epicatequina), B-3((+)-catequina-(4α→8)-(+)-catequina) e B-4 ((+)-catequina-(4α→8)-(–)-epicatequina)(Aron and Kennedy, 2008). A estrutura dos principais monômeros e dímeros de flavan-3-ol estão apresentados nas Figuras 1 e 2, respectivamente.



Monômeros de flavan-3-ols	R_1	R_2	R_3	R_4	R_5
(+)-catequina	H	OH	H	OH	OH
(-)-epicatequina	H	OH	OH	H	OH
(-)-epigalocatequina	OH	OH	OH	H	OH

Figura 1. Estrutura química dos principais monômeros de flavan-3-ol (Adaptado de Aron & Kennedy, 2008).



Dímeros de flavan-3-ols	R_1	R_2	R_3	R_4
Procianidina B1	OH	H	H	OH
Procianidina B2	OH	H	OH	H
Procianidina B3	H	OH	H	OH
Procianidina B4	H	OH	OH	H

Figura 2. Estrutura química dos principais dímeros de flavan-3-ol (Adaptado de Aron & Kennedy, 2008).

Resultados prévios do nosso grupo demostraram a possiblidade de utilização dos resíduos de vinificação, tanto de *V. vinifera* quanto de *V. labrusca*, para obtenção de compostos flavan-3-ol com importante atividade antioxidante (Scola *et al.*, 2010, Scola *et al.*, 2011). A partir destes resultados, selecionou-se o extrato de *V. labrusca* da variedade Bordo (por apresentar maior conteúdo de compostos fenólicos e melhor atividade antioxidante) para a realização deste trabalho.

1.2 Atividade biológica de polifenóis

Nos últimos anos, cientistas de diferentes áreas tem voltado sua atenção à procura de moléculas naturais na tentativa de desenvolver novos agentes terapêuticos. Os produtos naturais têm sido bastante pesquisados, já que são responsáveis por um número crescente de compostos que estão sendo testados em ensaios pré-clínico e clínico.

Os efeitos biológicos dos polifenóis são bem conhecidos. Estes compostos apresentam atividade antioxidante, anticarcinogênica, antiaterogênica, cardiopreventiva, antimicrobiana, antiviral, neuroprotetora e antiinflamatória (para revisão ver Ferguson, 2001, Ferguson & Philpott, 2008). Extratos de semente de *V. vinifera* mostraram atividade antiaterogênica, cardioprotetora, antimicrobiana, antiviral e anti-inflamatória (Gabetta *et al.*, 2000, Fan and Lou, 2004, Aron & Kennedy, 2008, Terra *et al.*, 2009).

Scola *et al*. (2010 & 2011) compararam a atividade antioxidante de diferentes extratos de resíduos de vinificação, entre eles, dois extratos oriundos de resíduos de *V. vinifera* (Cabernet Sauvignon e Merlot) e dois de resíduos de *V. labrusca* (Isabel e Bordo). Os extratos estudados mostraram significativa atividade antioxidante, tanto *in vitro* como *in vivo* e apresentaram, ainda, atividade anti-inflamatória *in vivo* (Anexos 1 e 2).

Os polifenóis também podem alterar a função cerebral melhorando o fluxo sanguíneo cerebral, modificando a permeabilidade da barreira hematoencefálica, ou a dinâmica dos neurônios e células gliais (Youdim *et al.*, 2004, Spencer, 2007, Ghosh and Scheepens, 2009, Schaffer & Halliwell, 2012) e alterando os níveis de neurotransmissores como a dopamina e de noradrenalina (Yoshitake *et al.*, 2010, Schaffer & Halliwell, 2012).

Os efeitos dos polifenóis no sistema nervoso central (SNC) tornam estas moléculas alvo de interesse no estudo de diversas doenças incluindo as desordens convulsivas. Esta patologia atinge cerca de 50 milhões de pessoas (WHO, 2011). No Brasil, estima-se que 1,8% da população apresenta a desordem convulsiva ativa (Noronha *et al.*, 2007). Essa doença é

caracterizada pela predominância de crises convulsivas espontâneas, recorrentes, com interrupções das funções normais do cérebro (Fisher *et al.*, 2005). Uma das peças fundamentais na fisiopatologia desta desordem é o estresse oxidativo, que possui reconhecido fator na propagação das convulsões (Costello & Delanty, 2004).

O estresse oxidativo pode ser resultante da depleção dos antioxidantes endógenos, perda de constituintes essenciais, como o cobre, ferro, zinco e o magnésio, e/ou exposição a agentes tóxicos (Halliwell & Gutteridge, 2007). O aumento de espécies reativas (ER) pode modificar a dinâmica celular, influenciando, inclusive, na liberação de neurotransmissores. Nas desordens convulsivas, as ER aumentam a concentração do neurotransmissor excitatório ácido aspártico e diminuem a liberação do neurotransmissor inibitório ácido γ-aminobutírico (Costello & Delanty, 2004, WHO, 2011).

Diferentes drogas têm sido prescritas à pacientes com diagnóstico de desordem convulsiva. No entanto, cerca de 30 a 40% destes pacientes não obtém uma resposta satisfatória (Sun *et al.*, 2010). Além disso, os medicamentos mais utilizados (fenitoína, carbamazepina, valproato, clonazepam e primidona) podem gerar efeitos colaterais importantes (Loscher & Potschka, 2005, López-Hernández *et al.*, 2005), indicando a necessidade de desenvolvimento de novas alternativas para o tratamento.

O estresse oxidativo pode estar relacionado à alteração do metabolismo energético causado por disfunções na cadeia transportadora de elétrons mitocondrial (Scola *et al.*, 2013). Diversas proteínas são alvos das ER, principalmente, as proteínas do complexo I, que são mais suscetíveis aos danos de nitração (Murray *et al.*, 2003). Nesta condição, modificações na estrutura e funcionalidade das proteínas, levam a diminuição da atividade do complexo I (Murray *et al.*, 2003) e ao aumento ainda maior da geração de espécies reativas (Beal, 2002, Murray *et al.*, 2003, Lee *et al.*, 2009), com consequente diminuição da produção de ATP.

Alterações nos níveis de expressão de proteínas da cadeia transportadora de elétrons foram reportadas em desordens convulsivas e psiquiátricas, associados ao estresse oxidativo

(Chuang *et al.*, 2010, Clay *et al*., 2010). No entanto, até o momento, não há estudos acerca do possível efeito dos polifenóis na modulação destas proteínas.

Os polifenóis, principalmente os compostos flavan-3-ol podem, ainda, reduzir o risco de formação e progressão de diferentes tumores (Sun *et al.*, 2006, Gerhauser, 2008), apresentando efeitos quimiopreventivos (Carlson *et al.*, 2008). Extratos de sementes de uva das variedades vinifera foram capazes de inibir o crescimento de tumores de mama e de pele em ratos e camundongos (Ye *et al.*, 1999, Singletary & Meline, 2001, Singh *et al.*, 2004).

A eficácia dos compostos monoméricos de flavan-3-ol também foi documentada no câncer de cólon (Engelbrecht *et al.*, 2007, Kaur *et al.*, 2008), onde o tratamento com estes compostos em células Caco-2 inativou a via de sinalização da fostaidilinositol-3-quinase, levando a diminuição da expressão das proteínas Bcl-2 (anti-apoptóticas) e induzindo a morte programada (Engelbrecht *et al.*, 2007, Kaur *et al.*, 2008). Além disso, o tratamento com extrato de *V. vinifera* aumentou a expressão das caspases e do fator de indução de apoptose em células de câncer de cólon (Dinicola *et al.*, 2010).

O câncer é umas das principais doenças crônicas e uma das maiores causas de morte no mundo. No ano de 2010, foram descritos 12 milhões de novos casos com morte de 7 milhões de pessoas (INCA, 2012). No Brasil, à exceção do câncer de pele do tipo não melanoma, os tumores de mama e de colo do útero são os principais tumores detectados na população feminina (INCA, 2012, WHO, 2012). O câncer de fígado ocupa a quinta posição na lista de tumores mais comuns, tanto em homens quanto em mulheres (WHO, 2012).

As neoplasias caracterizam-se pela proliferação celular anormal, exagerada e incontrolada, como resultado de danos aos mecanismos de regulação do ciclo celular e alteração gênica, modificando o crescimento e a diferenciação das células (Hanahan & Weinberg, 2011). Ademais, estas células possuem crescimento incontrolável, evasão do sistema de checagem celular e insensibilidade aos sinais inibitórios de crescimento (Shay & Roninson, 2004, Burzynski, 2005, Vattemi & Claudio, 2007, Hanahan & Weinberg, 2011).

As mutações são caracterizadas como o resultado das modificações do material genético das células primárias que pode levar ao desenvolvimento de células cancerígenas, o que, por sua vez, iniciará o processo de invasão e penetração nos tecidos adjacentes, e possível iniciação de metástases (Shay and Roninson, 2004, Burzynski, 2005, Vattemi & Claudio, 2007, Hanahan & Weinberg, 2011). O resultado de uma mutação são alterações na seqüência de bases do DNA, que, se replicada, será transmitida às futuras gerações, tornando-se permanente (Hanahan & Weinberg, 2011).

Com a inadequada função dos genes que regulam a proliferação e transformação celular, diversos processos de ativação de oncogenes e a omissão dos genes supressores de tumor podem desencadear o aparecimento de alterações fenotípicas nestas células, o que leva a adaptação, sobrevivência e a possibilidade de expansão. Ambos, oncogenes e os genes supressores de tumor, são responsáveis pela dinâmica celular. Alterações nestes mecanismos podem resultar em uma superexpressão de proteínas que propiciam a formação tumoral (Shay & Roninson, 2004, Vattemi & Claudio, 2007). A inibição da modulação gênica de supressores de tumor e/ou expressão de diferentes proteínas que conferem resistência para iniciação tumoral, tornaram-se um importante alvo para o desenvolvimento de novas drogas anticarcinogênicas.

Uma das principais maneiras de tentar evitar a proliferação tumoral é a indução da morte celular programada, também conhecida como apoptose. Este processo leva a autodestruição celular, considerado essencial no desenvolvimento e manutenção da homeostase (Lowe & Lin, 2000, Johnstone *et al.*, 2002). A apoptose, quando ativada, altera as características morfológicas celulares gerando o encolhimento celular, condensação da cromatina nuclear e sua fragmentação (Sun & Peng, 2009). Moléculas que modulam a via apoptótica podem representar alternativas promissoras para o tratamento do câncer.

Um importante fator de transcrição nos processos de regulação do ciclo de divisão celular é a proteína p53, responsável pela manutenção da integridade genética, e que faz a

verificação quanto à eventual ocorrência de uma mutação na sequência do código genético. Caso seja encontrada a existência de uma mutação, a p53 sinaliza diversas reações, ativando as proteínas de reparo, como a poli (ADP-ribose) polimerase (PARP), na tentativa de evitar danos ao DNA (Chen, 2011). A PARP é uma família de proteínas encontrada no núcleo das células e que está envolvida em diversos processos celulares, mas, principalmente, na reparação do DNA. O principal papel destas proteínas é sinalizar os danos de quebras de fita única do DNA para a maquinaria enzimática envolvida no reparo (Chen, 2011). Caso o dano seja incorrigível, cabe à proteína p53 impedir que a célula em questão complete a divisão celular (Roy *et al.*, 2005, Ghosh *et al.*, 2006). Este processo pode ocorrer através de duas vias, a via mitocondrial ou via intrínseca e a via extrínseca.

A via mitocondrial envolve a participação de diversas proteínas que são liberadas no citosol. A liberação do citocromo c resulta na ativação do fator de ativação de apoptose 1 (Apaf-1), que, em presença de trifosfato de adenosina (ATP), resulta na ativação da procaspase-9 iniciando o processo apoptótico (Zou *et al.*, 1997). No mecanismo independente da ativação de caspases, ocorre a liberação do fator de indução de apoptose (AIF) (Bidere *et al.*, 2003). Esta proteína encontra-se normalmente entre as membranas da mitocôndria, e quando liberada para o citosol, migra para o núcleo da célula onde é responsável pela condensação da cromatina e a degradação do DNA, induzindo a apoptose (Susin *et al.*, 1999, Hangen *et al.*, 2010).

Em sinergismo ao AIF, a proteína Bax, conhecida por induzir a apoptose através da interação com outros componentes pró-apoptóticos, forma canais transmembrana e contribui para regulação de canais preexistentes que permeabilizam a membrana mitocondrial externa, estimulando assim, outras proteínas apoptóticas, tais como BIM e BH3 pertencentes à subfamília de proteínas Bcl-2 (Jourdain & Martinou, 2009).

Já a via de morte extrínseca é caracterizada por sinalizações externas em vários receptores de membrana plasmática pertencentes à superfamília do fator de necrose tumoral (TNF), incluindo Fas (CD95), e a caspase-8 (Pitti *et al.*, 1996, Ashkenazi & Dixit, 1998).

Na busca por moléculas que apresentem mecanismos de ação novos e originais, os produtos naturais têm demonstrado ser uma importante fonte para o desenvolvimento de medicamentos. Tendo em vista os efeitos benéficos de sementes de uva já descritos na literatura, este trabalho deu continuidade a estudos prévios do nosso grupo, especificamente aos resíduos de vinificação de *V. labrusca*, variedade Bordo.

OBJETIVOS

2. OBJETIVOS

2.1 Objetivo Geral

Avaliar as atividades neuroprotetora, hepatoprotetora, genotóxica, mutagênica e citotóxica do extrato aquoso de sementes de resíduos de vinificação de *V. labrusca*, variedade Bordo, em diferentes modelos.

2.2 Objetivos específicos

- Estudar a atividade neuroprotetora e hepatoprotetora do extrato de *V. labrusca* em modelo de ratos Wistar tratados com a droga convulsiva pentilenotetrazol;
- Determinar a possível atividade genotóxica (ensaio Cometa) em sangue total de ratos tratados com o extrato de *V. labrusca*;
- Investigar a possível atividade mutagênica do extrato de *V. labrusca* em células eucarióticas da levedura *Saccharomyces cerevisiae*;
- Examinar a atividade do extrato de *V. labrusca* na modulação da expressão das proteínas do complexo I da cadeia transportadora de elétrons (NDUFV2, NDUFS7 e NDUFS8), em células de neuroblastoma humano (SH-SY5Y);
- Avaliar a citotoxicidade do extrato de *V. labrusca* nas linhagens celulares de câncer de fígado (HepG2) e de mama (MCF-7);
- Verificar o efeito do tratamento com o extrato de *V. labrusca* nos níveis de expressão das proteínas apoptóticas p53, Bax e AIF, PARP total e Her-2 nas linhagens HepG2 e MCF-7.

RESULTADOS E DISCUSSÃO

3. RESULTADOS E DISCUSSÃO

Os resultados e a discussão desta tese estão apresentados em forma de artigos científicos divididos em três capítulos. O primeiro capítulo teve como objetivo investigar o possível efeito neuroprotetor e hepatoprotetor do extrato aquoso de *V. labrusca* em ratos Wistar tratados com pentilenotetrazol. Alterações comportamentais, efeito genotóxico (ensaio cometa) e mutagênico (em células de *Saccharomyces cerevisiae*) também foram avaliados. O segundo capítulo mostra a ação do extrato de *V. labrusca* na expressão das proteínas do complexo I da cadeia transportadora de elétrons, em células de neuroblastoma humano (SH-SY5Y) tratadas com H_2O_2. Por fim, o terceiro capítulo mostra o efeito citotóxico do extrato de *V. labrusca* em linhagens de câncer de fígado (HepG2) e de mama (MCF-7), avaliando a modulação de proteínas apoptóticas (p53, Bax e AIF) e proteínas responsáveis pela proliferação celular (PARP e Her-2).

3.1 CAPÍTULO 1

Flavan-3-ol compounds prevent pentylenetetrazol-induced oxidative damage in rats without producing mutations and genotoxicity

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Flavan-3-ol compounds prevent pentylenetetrazol-induced oxidative damage in rats without producing mutations and genotoxicity

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HIGHLIGHTS

- We investigated the protective effects of administering flavan-3-ol extract in rats.
- The extract shows protective effects against neuronal and hepatic damage.
- The extract was shown to be non-genotoxic and non-mutagenic.
- This extract may be used to develop new therapeutic agent against seizures disorder.

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ABSTRACT

Seizure disorder is a chronic condition in the brain that affects approximately 50 million people worldwide. Oxidative stress plays a crucial role in the pathophysiology of this disorder and can cause neuronal injury. Approximately one in three treated patients suffers from seizures regardless of pharmacological intervention, which results in oxidative damage. The present study aims to investigate the possible protective effect of antioxidant-rich *Vitis labrusca* extract on pentylenetetrazol-induced oxidative damage in Wistar rats. Possible behavioral alterations, genotoxic and mutagenic effects of the extract were also evaluated. The results showed that *V. labrusca* extract provides a significant protective effect against oxidative damage to lipids and proteins induced by pentylenetetrazol in the cerebral cortex, cerebellum, hippocampus and liver of rats. Also, the extract did not alter locomotor behavior. Moreover, it was non-genotoxic and non-mutagenic. Our results suggest the possibility of using *V. labrusca* extract as a therapeutic agent to minimize neuronal damage associated with seizures.

1. Introduction

There has been an emerging interest in natural drugs in the past few years. Although several studies have reported that polyphenols improve brain function, their mechanism of action remains poorly understood. In fact, polyphenols were shown to be powerful antioxidants that can prevent reactive species formation by chelating trace elements involved in free radical production, scavenging reactive species, and protecting antioxidant defenses [19].

One of the main causes of cellular damage in the brain is oxidative stress. Reactive oxygen or nitrogen species (ROS or RNS) are directly involved in oxidative damage to proteins, lipids and DNA [19]. Protein oxidative damage can be induced through the reaction between protein and a hydroxyl radical, which introduces carbonyl groups [6]. Moreover, peroxynitrite and nitrous anhydride can cause lipid-peroxidation and DNA single/double-strand breaks that may result in cell death [15].

Excessive production of free radicals is involved in the pathophysiology of seizure disorder [8,10,30,38], where repeated seizures increase the production of ROS and RNS in the brain [8,10,30,38], increasing the likelihood of neuronal death [10,36,48] and subsequent neurodegeneration [38,48]. Furthermore, it has already been shown that seizures can produce oxidative damage in the liver [14]. Despite advances in diagnosis and treatments, the prognosis for patients with seizure disorder remains poor [40], and patients show considerable morbidity. For this reason, it is crucial to research new agents for the management of this disorder.

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Flavan-3-ol compounds [34,37] are polyphenol constituents of *Vitis labrusca*, which present considerable effects [34,35], and modulate cell functionality [12]. However, previous reports only examined a limited number of factors, including in vitro antioxidant and in vivo anti-inflammatory activities [34,35]. Furthermore, these properties raise the possibility that these compounds may be used as therapeutic agents for the prevention of oxidative damage in seizures. For this, the purpose of this study was to expand upon previous findings by investigating the protective effect of the *V. labrusca* winery seeds extract (VLE) against pentylenetetrazol (PTZ)-induced oxidative damage in Wistar rats. To determine possible protective effects of the VLE, oxidative damage and antioxidant defenses were evaluated in the cerebral cortex, cerebellum, hippocampus, and liver of rats. Possible behavioral alterations were also evaluated by the Open Field test. In addition, the possible genotoxic effect of VLE on lymphocytes of rats was analyzed and the mutagenic effect of VLE was evaluated using a recognized model of *Saccharomyces cerevisiae*.

2. Materials and methods

2.1. Chemicals

Pentylenetetrazol, thiobarbituric acid, 2,4-dinitrophenylhydrazine, 5,5′-dithiobis(2-nitrobenzoic acid), (−)-epinephrine, guanidine hydrochloride, hydrogen peroxide, and methyl methanesulfonate were purchased from Sigma–Aldrich. All other reagents (Merck and Hexapur) and solvents (Sigma–Aldrich) were of analytical grade.

2.2. Winery wastes seeds extract

Seeds from winery wastes of *V. labrusca* (*cv. Bordo*) were used in this study. The extract was obtained using 5 g of seeds/100 mL of distilled water, under reflux (100 °C; 30 min), filtered through a 0.45 μm pore filter (Millipore) and freeze-dried (Edward freeze dryer) [25]. The extract was solubilized in saline (0.9% NaCl) immediately before use. The major compounds of the extract and detailed methods are listed in Supplementary data.

2.3. Animals, treatments and behavior alterations

Experiments were carried out on 60 male Wistar rats weighing 250–300 g that were allowed free-access to food and water. The experiments were done in accordance with "Guide for the Care and Use of Laboratory Animals, DHEW, publication no. (NIH) 85-23, 1985" and approved by the local ethics committee. Animals were randomly assigned to one of 6 groups (10 animals/group): (1) saline through intraperitoneal (i.p.) injection; (2) 100 mg/kg (i.p.) of VLE; (3) saline plus 60 mg/kg (i.p.) of PTZ; (4) 10 mg/kg (i.p.) of VLE plus 60 mg/kg (i.p.) of PTZ; (5) 50 mg/kg of VLE plus 60 mg/kg (both i.p.) of PTZ; (6) 100 mg/kg of VLE plus 60 mg/kg (both i.p.) of PTZ. The extract or saline was given 30 min before PTZ administration.

Possible locomotor behavior alterations produced by VLE were evaluated using the Open Field test [11]. Anxiety, locomotion and exploratory activities were evaluated in the animals following treatment. The numbers of crossings of square areas of the floor, rearing frequency and fecal bolus were evaluated [21,44]. Measures of behavioral changes were analyzed for each rat 10 min prior to PTZ administration. After PTZ administration, convulsive behavior was analyzed for 30 min according to Racine's Scale [24]. Convulsion parameters and mortality were evaluated. Detailed methods are described in Supplementary data.

2.4. Preparation of tissues and protective effect of VLE

After 30 min of PTZ administration, the animals were euthanized by decapitation and blood and brain were collected. The cerebral cortex, cerebellum, hippocampus, and liver were homogenized. Damage to lipids was monitored by the formation of thiobarbituric acid reactive species (TBARS) [45]. Oxidative damage to proteins was measured by determining carbonyl groups based on the reaction with dinitrophenylhydrazine (DNPH) [23]. SOD activity was determined by measuring the inhibition of the rate of auto-catalytic adrenochrome formation. One unit was defined as the amount of enzyme that inhibits the rate of adrenochrome formation by 50% [5]. CAT activity was determined by the hydrogen peroxide (H_2O_2) decomposition rate. Values were expressed as μmol of H_2O_2 decomposed/min/mg of protein [1]. Determination of protein sulfhydryl content was based on the reaction with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) [2]. Protein concentration was determined by the Bradford method [7]. Detailed methods are described in Supplementary data.

2.5. Genotoxicity assay

Comet assay [9,27] was performed to assess potential genotoxic effects of VLE on lymphocytes of VLE-treated rats and controls. Methyl methanesulfonate (MMS, 8×10^{-5} M) was used as DNA damage positive control. Images of 100 randomly selected cells were analyzed from each sample. The damage was visually scored according to tail size into five classes, from no tail (0) to maximal (4) long tail, resulting in a single DNA damage score for each treatment. Therefore, damage index (DI) ranged from 0 (all cells with no tail, 100 cells × 0) to 400 (all cells with maximally long tails, 100 cells × 4). Detailed methods are described in Supplementary data.

2.6. Mutagenic effect of VLE

Saccharomyces cerevisiae XV185-14c strain was applied to assess potential mutagenic effects of VLE. Cells were grown and incubated in different VLE concentrations (0.05, 0.5 and 5 mg/mL). Survival and induction of mutations for *LYSINE*$^+$, *HISTIDINE*$^+$ or *HOMOSERINE*$^+$ was performed [20,33,43,49]. H_2O_2 (75 mM) was used as a positive control. Detailed methods are described in Supplementary data.

2.7. Statistical analysis

All measurements were performed at least in triplicate. Values were averaged and expressed along with the standard error of mean (SEM), except for the genotoxic and mutagenic assays, which were presented with standard deviation (SD) values. Results were subjected to one-way analysis of variance (ANOVA) and Tukey's post hoc test. To evaluate the significance of the difference between controls and VLE treated rats for the frequency of DNA damage, results were submitted to the independent measures *t*-test ($p < 0.05$) using SPSS 19.0 software.

3. Results

3.1. VLE prevented PTZ-induced mortality

VLE did not induce mortality in the highest concentration assayed (100 mg/kg). Moreover, PTZ caused a progressive increase in seizure intensity, inducing mortality around 40% of PTZ-treated animals. VLE was able to prevent mortality in all concentrations assayed (10, 50 and 100 mg/kg) (Supplementary Fig. 3).

3.2. VLE presented substantial protective effects

To evaluate the possible protective effect of the VLE, damage to lipids and proteins was quantified. PTZ treatment induced a significant increase in TBARS, carbonylated proteins, and the activity of SOD in the cerebral cortex, cerebellum, hippocampus and liver of rats. PTZ treatment also increased the activity of CAT in the cerebellum and hippocampus, but not for the cerebral cortex. However, PTZ treatment did not affect protein sulfhydryl amounts (Table 1).

VLE (10 and 50 mg/kg) was able to reduce the PTZ-induced increase in TBARS (in the cerebellum) and carbonylated proteins (in the cerebellum, hippocampus and liver). Both of these concentrations ameliorated the PTZ-induced increase in SOD activity in the liver while further increasing CAT activity. At a higher concentration, VLE reduced PTZ-induced increase in TBARS and carbonylated protein levels in all tissues. However, VLE (100 mg/kg) significantly reduced the SOD and CAT activity in the cerebral cortex, and the activity of SOD in hippocampus (Table 1). Moreover, VLE (100 mg/kg) alone and VLE (100 mg/kg) + PTZ increased protein sulfhydryl content above controls in the cerebellum and hippocampus.

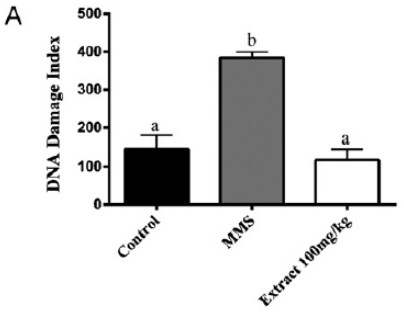
3.3. VLE did not alter locomotor behavior

Behavioral analyses were conducted using the Open Field test. VLE did not affect the number of total crossings, rearings or fecal bolus of rats. In the group treated with 10, 50 and 100 mg/kg of extract, we observed a trend toward decreased fecal bolus (Supplementary Fig. 4).

3.4. VLE did not present genotoxic effects

The genotoxic effect of VLE was assayed on lymphocytes. DNA damage was evaluated first by the overall score on the DNA damage index (DI) (Fig. 1A), and second by the frequency of cells in each class of DNA damage (Fig. 1B). The DI and the DNA class damage frequency showed that VLE in the highest concentration did not induce genotoxicity when compared to MMS that was used as a positive control. For DNA class damage frequency, majority of the cells in the VLE group were found in classes 0 and 1 of DNA damage for 100 mg/kg of VLE, followed by classes 2, 3 and 4 (Fig. 1B). In addition, the distribution of cells for the VLE group did not show a significant difference when compared to control, demonstrating non-genotoxicity.

A



B

Frequency (%) of DNA damage					
	Undamaged cells	class one	class two	class three	class four
Control	13.60±1.32	40.80±2.76	23.30±1.69	12.70±1.09	9.60±0.70
MMS	0.00±0.00#	0.00±0.00#	5.86±0.83#	9.86±0.74	84.28±3.77#
Extract 100mg/kg	45.78±2.94#	25.57±2.75#	15.22±1.69#	8.91±0.44#	4.52±0.31#

Fig. 1. (A) DNA damage index by comet assay in lymphocytes of rats treated with *V. labrusca* extract. (B) Frequency (%) of different classes of DNA damage (comet assay) in controls and *V. labrusca* extract-treated group. Cells were assessed visually and received scores from 0 (undamaged) to 4 (maximally damaged) according to the size and shape of the tail. Results represent average values ± SD. Superscript letters (A) indicate significant differences according to ANOVA and Tukey's post hoc test ($p \leq 0.01$). # (B) Statistical significance for VLE and MMS-treated groups against control using independent *t*-test ($p < 0.05$). MMS, methyl methanesulfonate.

Table 1
Oxidative stress markers in brain structures of rats treated with *V. labrusca* extract.

Structure	Groups	TBARS (nmol TBARS/mg protein)	Carbonylated proteins (nmol DNPH/mg protein)	Superoxide dismutase (U SOD/mg protein)	Catalase (μmol of H_2O_2 decomposed/min/mg protein)	Protein sulfhydryl content (μmol DTNB/mg protein)
CC	Control	6.52 ± 0.98	6.53 ± 0.19	30.08 ± 3.60	24.99 ± 2.23	0.25 ± 0.03
	Extract (100 mg/kg)	3.38 ± 0.43	2.23 ± 0.38	7.50 ± 0.52*	8.45 ± 0.94*	0.21 ± 0.01
	PTZ	20.00 ± 3.01*	18.42 ± 2.85*	53.78 ± 4.31*	29.29 ± 3.14	0.15 ± 0.02
	Extract (10 mg/kg) + PTZ	17.92 ± 1.11*	18.54 ± 1.34*	42.16 ± 3.01	26.07 ± 2.75	0.19 ± 0.05
	Extract (50 mg/kg) + PTZ	15.03 ± 1.96*	13.29 ± 0.85*	41.59 ± 6.24	28.51 ± 2.59	0.19 ± 0.02
	Extract (100 mg/kg) + PTZ	8.28 ± 0.63#	4.61 ± 0.96#	41.31 ± 4.94	40.41 ± 5.13	0.22 ± 0.02
CE	Control	7.77 ± 0.63	2.25 ± 0.37	14.49 ± 2.49	18.56 ± 3.34	0.15 ± 0.02
	Extract (100 mg/kg)	4.98 ± 0.99	2.43 ± 0.51	10.94 ± 0.94	10.72 ± 0.83	0.25 ± 0.02*
	PTZ	31.33 ± 2.65*	9.94 ± 2.14*	50.32 ± 3.03*	32.41 ± 4.86*	0.10 ± 0.01
	Extract (10 mg/kg) + PTZ	24.30 ± 1.49*,#	3.88 ± 0.67#	41.67 ± 2.30*	31.30 ± 1.26*	0.11 ± 0.01
	Extract (50 mg/kg) + PTZ	24.41 ± 0.86*#	3.54 ± 1.15#	34.56 ± 3.51*	35.29 ± 3.43*	0.18 ± 0.01
	Extract (100 mg/kg) + PTZ	15.75 ± 1.43*,#	1.96 ± 0.37#	29.39 ± 2.64*	41.46 ± 4.28*	0.27 ± 0.02*,#
HIP	Control	4.15 ± 0.67	3.19 ± 0.38	14.85 ± 2.84	24.36 ± 3.23	0.25 ± 0.02
	Extract (100 mg/kg)	4.07 ± 0.79	1.80 ± 0.29	14.63 ± 1.68	4.92 ± 0.39*	0.21 ± 0.01
	PTZ	22.80 ± 2.23*	7.24 ± 0.83*	46.53 ± 3.51*	51.43 ± 7.57*	0.15 ± 0.02
	Extract (10 mg/kg) + PTZ	18.05 ± 0.72*	3.44 ± 0.50#	43.89 ± 3.85*	51.16 ± 2.41*	0.16 ± 0.03
	Extract (50 mg/kg) + PTZ	22.24 ± 1.00*	3.08 ± 0.38#	39.67 ± 4.35*	51.52 ± 2.40*	0.16 ± 0.02
	Extract (100 mg/kg) + PTZ	14.61 ± 1.90*,#	2.80 ± 0.25#	49.72 ± 4.01*	48.86 ± 4.34*	0.27 ± 0.05#
LI	Control	3.49 ± 0.05	40.53 ± 1.44	6.42 ± 0.80	13.90 ± 2.91	0.50 ± 0.09
	Extract (100 mg/kg)	4.53 ± 0.51	15.14 ± 1.17*	5.65 ± 0.48	8.69 ± 0.77	0.49 ± 0.12
	PTZ	14.75 ± 2.51*	190.31 ± 8.21*	55.01 ± 6.33*	101.55 ± 6.46*	0.36 ± 0.08
	Extract (10 mg/kg) + PTZ	12.85 ± 1.08*	52.53 ± 5.91*,#	34.03 ± 3.36*,#	100.75 ± 4.45*	0.36 ± 0.04
	Extract (50 mg/kg) + PTZ	12.28 ± 0.86*	20.68 ± 4.35*,#	33.91 ± 4.82*,#	140.82 ± 7.07*,#	0.36 ± 0.03
	Extract (100 mg/kg) + PTZ	7.16 ± 0.60#	9.94 ± 1.03#	38.05 ± 0.40*,#	143.68 ± 9.98*,#	0.55 ± 0.09

Data are mean ± SEM. CC, cerebral cortex; CE, cerebellum, HIP, hippocampus; LI, liver.
* Values significantly different from control group.
# Values significantly different from pentylenetetrazol (PTZ) group. ANOVA and Tukey's post hoc test ($p \leq 0.05$) was used.

Table 2
Induction of point (his1-7 and lys1-1) and frameshift (hom3-10) mutations after treatment with *V. labrusca* extract in *S. cerevisae* XV185-14c strain.

Treatments	Survival (%)	*His*$^+$ revertants	*Lys*$^+$ revertants	*Hom*$^+$ revertants
Saline	100^{a*}	5.36 ± 0.57^a	1.67 ± 0.18^a	1.01 ± 0.11^a
75 mM H_2O_2	40^b	44.10 ± 2.37^b	5.47 ± 1.09^b	13.15 ± 1.00^b
Extract 0.05 mg/mL	100^a	3.57 ± 0.24^a	1.65 ± 0.03^a	0.79 ± 0.01^a
Extract 0.5 mg/mL	100^a	2.91 ± 0.18^a	1.71 ± 0.10^a	0.5 ± 0.03^a
Extract 5 mg/mL	100^a	4.14 ± 0.01^a	1.82 ± 0.01^a	1.11 ± 0.01^a

Negative control was saline, and positive control was 75 mM H_2O_2. *His*$^+$, histidine; *Lys*$^+$, lisyne; and *Hom*$^+$, homoserine. Data are mean ± SD values.
* Superscript letters indicate significant differences according to ANOVA and Tukey's post hoc test ($p \leq 0.01$).

3.5. VLE did not produce mutagenic effects

S. cerevisiae cells in the exponential growth phase were used to verify the protective effect of VLE against H_2O_2-induced DNA mutation. VLE did not induce locus non-specific, locus-specific or frameshift mutations in the XV185-14c strain in the different concentrations analyzed (0.05–5 mg/mL) (Table 2). When compared to H_2O_2 treatment, VLE pre-treatment enhanced cell survival and avoided mutations in all concentrations. In addition, VLE alone did not induce toxicity or mutagenic effects in *S. cerevisiae* in any of the concentrations assayed.

4. Discussion

PTZ-induced seizure model is a widely used animal model which affect the concentration of different neurotransmitters in the brain [41] as well as disrupting the blood–brain barrier (BBB) and impairing brain functions [24,29,42]. Several biological mechanisms by which seizures induce the formation of free radicals include the activation of n-methyl-D-aspartate receptors, adverse changes in patterns of synaptic transmission, increase in the effect of glutamate, and diminution of inhibitory influences on membrane activity [10].

The link between seizures and oxidative stress has been widely demonstrated [8,10,30,31,38]. In fact, in our study PTZ was found to significantly induce mortality and increase oxidative stress in rats. To explore the effect of VLE in PTZ-induced oxidative damage, markers of oxidative stress were measured. VLE (100 mg/kg) had a protective effect against PTZ-induced oxidative stress, as demonstrated by decreased lipid and protein damage in the cerebral cortex, cerebellum, hippocampus and liver of rats. Rodrigues et al. [31] found similar protective effects against PTZ-induced oxidative stress in lipids and proteins using grape juice, which is a rich source of catechin, for the same brain structures. Several other studies also found antioxidants such as α-tocopheryl-L-ascorbate-2-o-phosphate diester [47], lipoic acid [26], erdostein [22], isopulegol [39], and most recently epigallocatechin-3-gallate [46], to reduce seizure-induced oxidative stress. Moreover, VLE alone increased sulfhydryl content in the cerebellum. This effect of VLE was also seen in animals treated with the highest concentration of PTZ, where the extract was able to protect against PTZ-induced oxidation of sulfhydryl groups.

To examine the mechanism of action of the extract, the activity of SOD and CAT, which are enzymes involved in the conversion of superoxide anion into water [16,28], was measured. PTZ alone produced an increase in the activity of SOD and CAT, suggesting an increase in oxidative stress and subsequent increase in the production of antioxidant enzymes to combat this assault [18]. However, the addition of VLE did not have an effect on PTZ-induced increase in enzymatic activity in the brain while having different effects on PTZ-induced changes in SOD (decreased) and CAT (increased) in the liver. Interestingly, when PTZ is not administered, VLE (100 mg/kg) decreases SOD and CAT activities in the cerebral cortex and SOD activity in the hippocampus when compared to control. Possibly, VLE is decreasing the formation of superoxide anions by inhibiting enzymatic systems involved in ROS formation [17], thus reducing the need for antioxidant enzymes. Furthermore, it should be noted that these results demonstrate the effect of acute administration of the extract on SOD and CAT activities. This effect may be enhanced if the extract is administered for a longer period of time.

Polyphenols enter the brain at measurable levels (0.14 nmol/g tissue–0.57 ng/g tissue), and can alter brain function by improving cerebral blood flow, altering the BBB, and directly modifying the activity of neurons and glial cells [32]. This suggests that polyphenols present in VLE may be sufficient for the modulation of cellular dynamics promoting protection against PTZ-induced death. Surprisingly, all VLE concentrations were able to prevent mortality induced by PTZ. Additionally, in the liver, where the bioavailability of VLE is much greater, we saw a more pronounced modulation of antioxidant enzymes, resulting in higher protection against PTZ-induced oxidative damage when compared to the brain. Because oxidative stress plays a pivotal role in cellular injury [8,13,19], compounds that minimize this effect could be used to reduce seizure-induced oxidative damage in patients with seizure disorders [30].

Importantly, VLE was also shown to be non-genotoxic and non-mutagenic. Moreover, Bagchi et al. [4] reported that grape seed extract presented protection against DNA fragmentation. This suggests that VLE compounds might act synergistically to prevent oxidative damage to DNA during seizures. For DNA class damage frequency, it was possible to observe a significant difference after VLE treatment, which showed the protective effect of VLE in preserving DNA integrity. In addition, the emotional stability of rats was assessed using the Open Field method. This is a classic model to assess behavior that is sufficiently sensitive for the detection of the effects of psychostimulants or depressants [21,44]. VLE alone did not present alterations on the behavior of the animals, suggesting that it may be able to exert its protective effects without interfering with emotional stability. This suggests that VLE may be used as a therapeutic agent. It was also reported that extracts from grape seeds are safe for human consumption [3].

Limitations of this study include the short duration in which the extract was administered to the animals. The effect of the extract may be enhanced if administered for a longer period of time prior to PTZ treatment, which can be examined in future studies. Lastly, this study only examined a limited number of factors that are affected by or involved in the modulation of oxidative stress. Examining a broader range of factors, such as changes in the concentrations of neurotransmitters, the expression patterns of specific proteins, and alterations in DNA will help to further elucidate the mechanism of the VLE.

5. Conclusion

To summarize, the development of efficacious interventions for the treatment and management of neurologic disorders has been garnering much interest. VLE provides a significant protective effect against oxidative damage to lipids and proteins induced by pentylenetetrazol in the brain and liver of rats. VLE was also shown to be non-genotoxic, non-mutagenic and did not cause behavioral

alterations. Although further studies are needed to fully examine the mechanism of action of VLE, the results of this study demonstrate the potential of VLE to be used as a therapeutic agent to protect against damage induced by recurrent seizures.

Conflicts of interest

Authors declare no conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neulet.2012.11.056.

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Material suplementar

Suppl. 1. Full Methods

Winery wastes seeds extract

Seeds from winery wastes of *V. labrusca* (*cv. Bordo*) were removed from vinification tanks five days after beginning of fermentation. Seeds were manually separated from the rest of the winery wastes; the extracts were obtained using 5 g of seeds/100 mL of distilled water, under reflux (100 °C) for 30 min, filtered through a 0.45 µm pore (catalog number SFGS 047LS, Millipore Corp., Brazil) and freeze-dried (Edward freeze dryer, Brazil) [25]. The extract was solubilized in saline (0.9% NaCl) immediately before use. The major compounds of the extract are listed in Suppl. 2. No alkaloids, saponins, or terpenoids were found in the extract.

Open Field Test

In order to assess if *V. labrusca* extract administration could alter the behavioral parameters, treated rats were evaluated through the open field test. Anxiety, locomotion and exploratory activities were evaluated in the animals following the conclusion of the treatment (30 minutes). Experiments were carried out in a noise-free room. Rats were placed in the center of a square arena (50 x 50 x 50 cm) divided into 25 squares. Measures of behavioral changes in the open field apparatus were analyzed for each rat for 10 min prior to PTZ administration. The number of line crossing, rearing and fecal bolus during exploration were measured and recorded manually [11, 21, 44].

PTZ-induced seizure in acute model

After the injection of PTZ, animals were placed in individual plastic boxes, and convulsive behavior was analyzed for 30 min according to Racine's Scale [24]. Convulsion

parameters and mortality were evaluated for each rat by 2 trained observers blind to the treatment status.

Tissues preparation and protective effect of VLE

After 30 minutes of PTZ administration, animals were euthanized by decapitation without anesthesia, blood was collected and the brain was rapidly excised in a Petri dish and placed on ice. Cerebral cortex, cerebellum, hippocampus, and liver were dissected, weighed and kept chilled until homogenization, which was performed in 1.5% KCl using a ground-glass-type Potter-Elvehjem homogenizer. The homogenates were centrifuged (800 x *g*) for 10 min at 4°C; pellets were discarded, and supernatants were frozen at -80°C. Analyses of oxidative markers included quantification of oxidative damage to lipids, proteins, and activity of antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT). Protein sulfhydryl content was assessed as a non-enzymatic cellular defense. Oxidative damage to lipids was monitored by the formation of thiobarbituric acid reactive species (TBARS) [45], which has been widely adopted as a sensitive method for measuring lipid peroxidation. First, 1000 µL of 5% trichloroacetic acid was added to 250 µL of supernatants and centrifuged at 7000 x *g* for 10 min. Then, 1000µL of sulfuric acid (3 M) was mixed with 1000 µL of thiobarbituric acid solution. Reaction mixture was incubated in a boiling water bath for 15 min and cooled to room temperature. Then, 3500 µL of n-butanol was added, and the samples were centrifuged at 7000 x *g* for 5 min. The absorbance was read at 532 nm. Results were expressed as nmol of TBARS/mg of protein. Oxidative damage to proteins was measured by determining carbonyl groups based on the reaction with dinitrophenylhydrazine (DNPH) [23]. For this assay, 200 µL of DNPH (10 mM) or 200 µL of HCl (2 M) for control were added to 50 µL of supernatant. The reaction mixture was incubated in the dark for 30 min and vortexed every 10 min. Then, 250 µL of 20 % trichloroacetic acid was added and the samples were centrifuged at 4000 x *g* for 8 min. The supernatant was discarded, and the pellet was washed 3 times with ethanol-ethyl acetate (1:1) to remove free reagent. Samples were centrifuged, and pellets were

re-suspended in 600 µL of guanidine solution (6 M) at 37°C for 15 min. Absorbance was read at 365 nm. Results were expressed as nmol of DNPH/mg of protein. SOD activity [5] was determined by measuring the inhibition of the rate of auto-catalytic adrenochrome formation at 480nm in a reaction medium containing 1 mM adrenaline (pH 2.0) and 50 mM glycine (pH 10.2). This reaction was conducted at 30^{o}C for 3 min. The results were expressed as SOD units/mg of protein. One unit was defined as the amount of enzyme that inhibits the rate of adrenochrome formation by 50%. CAT activity [1] was determined by the hydrogen peroxide (H_2O_2) decomposition rate. Briefly, 20 µL of the sample was added to 2910 µL phosphate buffer (pH 7.4) plus 70 µL of H_2O_2 (3 mM freshly diluted) and read spectrophotometrically at 240 nm. Values were expressed as µmol of H_2O_2 decomposed/minute/mg of protein. Determination of protein sulfhydryl content [2] was based on the reaction with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), and the absorbance was measured spectrophotometrically at 412 nm. Briefly, 0.1 mM DTNB was added to 120 µL of supernatant. This was followed by 30 min incubation at room temperature in the dark. Sulfhydryl content is inversely correlated with oxidative damage to proteins. Results are expressed as µmol of DTNB/mg of protein. Protein concentration was determined by the Bradford method [7] using bovine serum albumin as standard.

Genotoxicity assay

Single cell gel electrophoresis or comet assay was performed using a modified method [9, 27] to assess potential genotoxic effects of VLE on lymphocytes separated from the blood using Histopaque®. Slides were prepared by mixing 20 µl of cell suspension and 80 µl low melting point agarose (0.75%). The mixture was poured onto a frosted microscope slide coated with normal melting point agarose (1%). After solidification, the coverslip was removed and the slides were placed in lysis solution (2.5 M NaCl, 100 mM EDTA and 10 mM Tris, pH = 10.0-10.5, with freshly added 1 ml Triton X-100 and 10% DMSO) for 24 h.

Subsequently, the slides were incubated in freshly made alkaline buffer (300 mM NaOH and 1 mM EDTA, pH = 12.6) for 20 min. DNA was electrophoresed for 20 min at 25 V (0.9 V/cm) and 300 mA, and the buffer was neutralized with 0.4 M Tris (pH = 7.5). Finally, DNA was stained with silver nitrate, and the slides were coded for blind analysis. Images of 100 randomly selected cells (of four replicated slides) were analyzed from each sample. Damages were visually scored according to tail size into five classes, from no tail (0) to maximal (4) long tail, resulting in a single DNA damage score for each treatment. Therefore, group damage index (DI) ranges from 0 (all cells with no tail, 100 cells ×0) to 400 (all cells with maximally long tails, 100 cells ×4). Methyl methanesulfonate (MMS, 8×10^{-5} M final concentration) was used as control for DNA damage.

Mutagenic effect of VLE

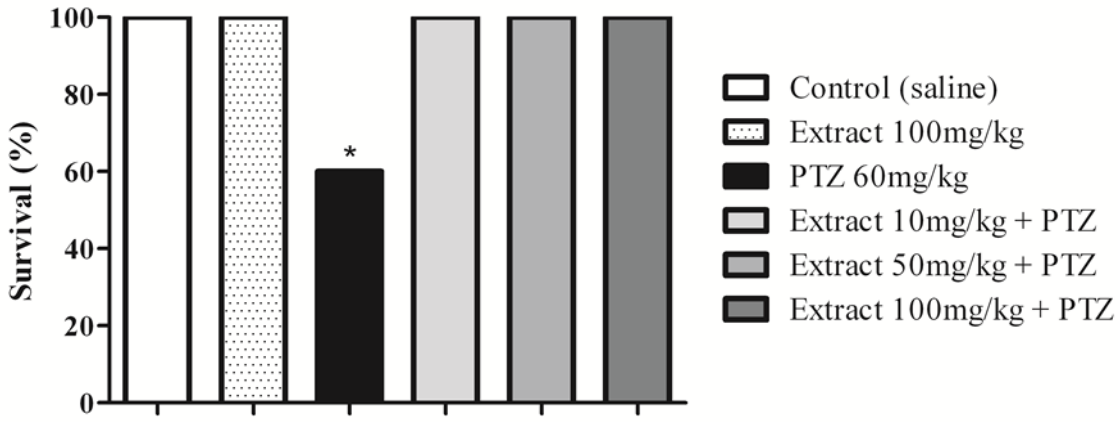
XV185-14c strain *(MATα, ade2-2, arg4-17, his1-7, lys1-1, trp5-48, hom3-10)* kindly provided by Dr. R.C. Von Borstel (Genetics Department, Alberta University, Canada) was used for the mutagenicity assay. Rich liquid medium (YPD) was used for routine growth. The minimal medium (MM) contained 0.67% yeast nitrogen base with no amino acids, 2% glucose, 2% bacto-agar and 0.25% $(NH_4)_2SO_4$. Synthetic complete medium (SC) consisted of MM supplemented with 2% adenine, 5% lysine, 1% histidine, 2% leucine, 2% methionine, 2% uracil, 2% tryptophan and 24% threonine w/v. Deficient medium lacking lysine (SC-lys), histidine (SC-his), or homoserine (SC-homo) was used for mutagenesis determination. A solution of 0.9% NaCl was employed to dilute cell suspensions. A 2 x 10^8 cells/mL suspension (stationary phase) of XV 185-14c yeast strain was grown until the exponential phase and then incubated for 2 h at 28°C in different *V. labrusca* extract concentrations (0.05, 0.5 and 5 mg/mL). Survival was determined in synthetic medium SC (3-5 days, 28°C), and mutation induction (*LYSINE*$^+$, *HISTIDINE*$^+$ or *HOMOSERINE*$^+$ *revertants*) was performed on corresponding deficient medium (7-10 days, 28^{o}C). While *his1-7* is a non-suppressible

missense allele in which reversion of the mutation must occur within the locus where the mutation originated, *lys1-1* is a suppressible ochre nonsense mutant allele [20] that can be reverted either by locus-specific or forward mutations in a suppressor gene [43]. Both mutations at the lys1-1 locus were differentiated [33]. It is believed that *hom3-10* contains a frameshift mutation, due to its response to several diagnostic mutagens [49]. Plating was done in triplicate for each dose. H_2O_2 (75 mM) was used as positive control.

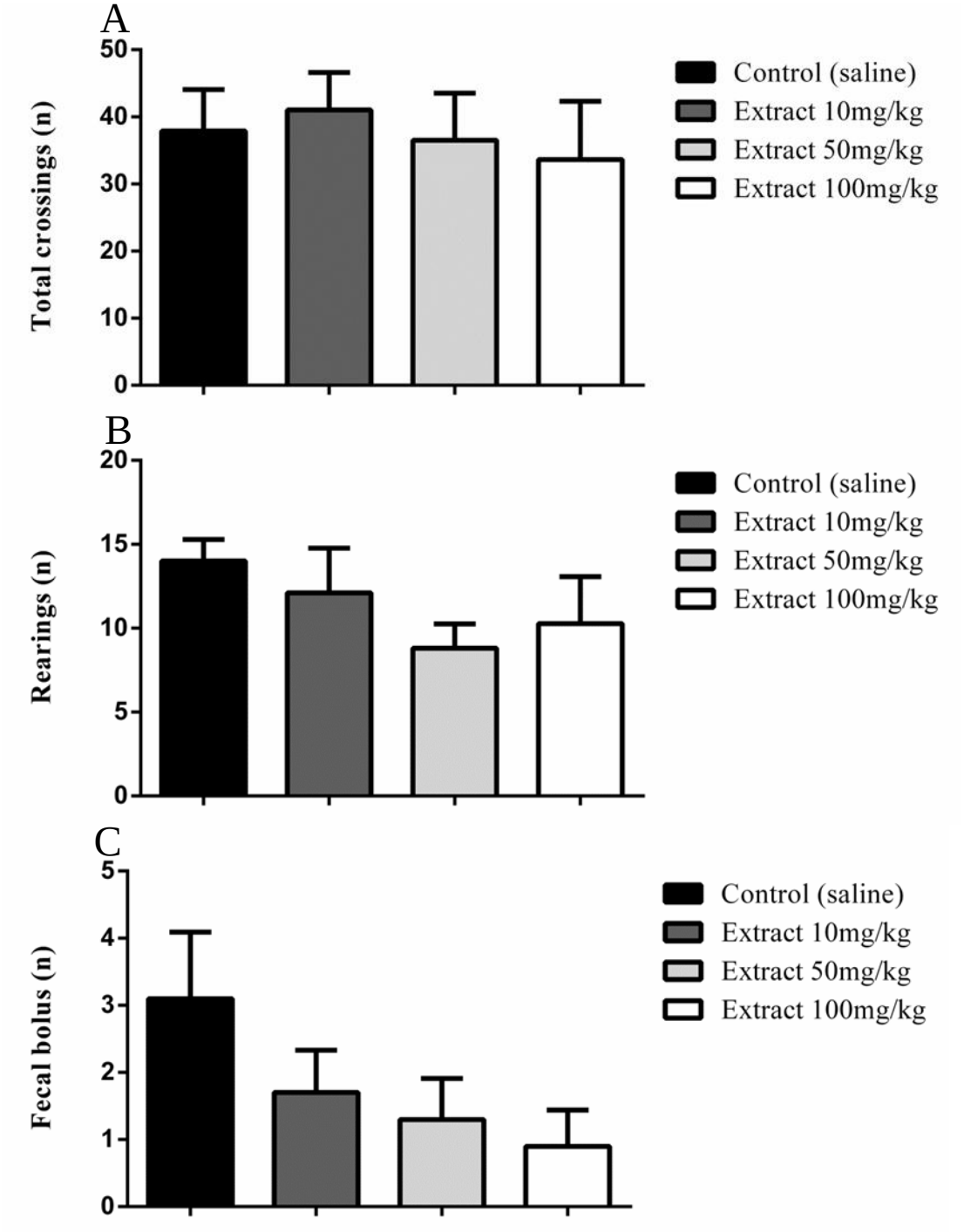
Suppl. 2. Total polyphenol content and major compounds in the *V. labrusca* extract

		Major compounds (mg/L)							
	TPC (mg/L of CT)	CT	ECT	EGC	B1	B2	B3	B4	GA
Extract	744.89±3.13	169.26±0.92[a*]	168.86±2.82[a]	8.96±0.05[b]	22.42±0.51[c]	19.75±0.17[d]	17.45±0.01[e]	1.85±0.12[f]	12.98±0.54[g]

Results represent average values ± S.D. [*]Superscript letters indicate significant differences according to one-way ANOVA and Tukey´s post-hoc test ($p \leq 0.05$) between major compounds. TPC, total phenolic content; CT, catechin; ECT, epicatechin; EGC, epigallocatechin; B1, procyanidin B1; B2, procyanidin B2; B3, procyanidin B3; B4, procyanidin B4; GA, gallic acid, data adapted from Scola *et al.* [34].



Suppl.3. Effect of different doses of *V. labrusca* extract in preventing PTZ-induced mortality. Data are expressed as mean (n=10). Fisher's exact test ($p<0.01$) was followed by Holm-Sidak post-hoc to compare treatments against saline control (*$p \leq 0.01$).



Suppl.4. Effect of different doses of *V. labrusca* extract on: A) number of total crossings, B) number of rearing, and C) number of fecal bolus [10 mg/kg (p=0.523), 50 mg/kg (p=0.305), and 100 mg/kg (p=0.142), respectively]. Data are expressed as mean ± S.E.M. Parametric distribution of the data was assessed using the Kolmogorov-Smirnov test. All data were found

to be parametrically distributed. *Significant difference according to one-way ANOVA and Tukey´s post-hoc test ($p \leq 0.05$).

3.2 CAPÍTULO 2

Vitis labrusca ***extract decreases suppression of complex I proteins:***

a source of therapeutic agents

Artigo a ser submetido.

Vitis labrusca extract decreases suppression of complex I proteins: a source of therapeutic agents

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One of the prominent hypotheses regarding the pathophysiology of neurologic diseases, such as seizure disorders and psychiatric disorders is increased oxidative stress and mitochondrial dysfunction, as shown in studies demonstrating reduced production of adenosine triphosphate (ATP) (1, 2), increased markers of oxidative damage (3), and polymorphism and reduced expression of genes encoding mitochondrial proteins (1, 4). Although there is a critical need for the development of more efficacious treatments for these disorders, progress has been limited due to a lack of understanding of their pathogenesis. Recently, a commentary in Biological Psychiatry (5) addressed differences in the expression of complex I genes, where decreased expression of genes specifically involved in the electron transfer process was reported.

Complexes I through V of the mitochondria are responsible for the generation of ATP, where ATP synthase utilizes the proton gradient produced by the coupling between the transfer of electrons and the pumping of protons (3, 6). Electrons can escape during this process to react with oxygen to create the superoxide anion, with complex I being a major site of free radical production (7). Complex I consists of 2 major modules, where the hydrophilic arm is the hydrogenase module responsible for the transfer of electrons, and the hydrophobic arm is the transporter module. Within the hydrogenase module, the transfer of electrons from flavin mononucleotide (FMN) to ubiquinone is performed by eight iron–sulfur clusters, where FMN and N3 are located in NDUFV1, N1a in NDUFV2, N1b, N4, N5, and N7 in NDUFS1,

N6a and N6b in NDUFS8, and N2 in NDUFS7 (5). On the other hand, the transporter module is largely responsible for the pumping of protons across the inner mitochondrial membrane (8).

Scola et al. (5) reviewed microarray studies and reported that 8 genes were found to be altered in bipolar disorder (BD), where the expression of iron-sulfur cluster-containing subunits within the hydrophilic arm were shown to be reduced, suggesting that patients with BD could be more prone to having a dysfunction in the electron transfer process. More specifically, NDUFV2, which is the first step in the electron transfer process, NDUFS8, which controls electron relay, and NDUFS7 responsible for the reduction of ubiquinone to ubiquinol, were found to be downregulated in BD. Considering the complexity of mitochondrial complex I and its significance for neurologic disorders, the aim of this report was to examine the ability of *Vitis labrusca* extract (VLE) to prevent oxidative damage to complex I proteins. VLE constituents are polyphenols flavan-3-ol compounds (9, 10), which present considerable effects (9, 10). However, previous reports only examined a limited number of factors, including *in vitro* and *in vivo* antioxidant and *in vivo* anti-inflammatory activities (11). Furthermore, these properties raise the possibility that these compounds may be used as therapeutic agents for the prevention of oxidative damage in neurologic disorders. For this, human neuroblastoma cells (SH-SY5Y) were treated with VLE (50ng/mL) for 3 days prior to the induction of oxidative damage with hydrogen peroxide (H_2O_2; 5, 10 and 50µM – 30 min), and protein levels of NDUFV2, NDUFS8 and NDUFS7 were measured using the immunoblotting method (12).

Following H_2O_2 treatment, our results showed decreased protein levels of NDUFV2, NDUFS8 and NDUFS7 (Figure 1A), which were reported to have altered expression levels in patients with BD by Scola et al (5). Importantly, pre-treatment with VLE resulted in a significant prevention of H_2O_2-induced downregulation in the protein levels of NDUFV2, NDUFS8 and NDUFS7 (Figure 2A).

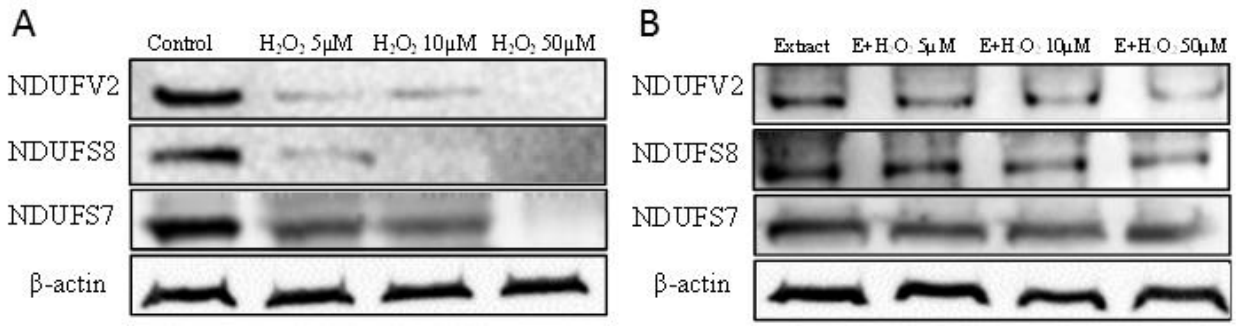

Figure 1. Immunoblotting showing altered expression of complex I proteins, NDUFV2 (ab87980), NDUFS8 (ab96123) and NDUFS7 (ab127051) in Neuroblastoma cells (ATCC CRL-2266). (**A**) Cells were treated with different concentrations of hydrogen peroxide (5, 10 and 50μM). (**B**) Cells were treated with VLE (50ng/mL) for 3 days prior to the induction of oxidative damage with hydrogen peroxide (30 min). Total cellular proteins of H_2O_2, VLE, and VLE+H_2O_2–treated cells were separated and electro-transferred onto nitrocellulose membrane. β-actin served as loading control. Specific secondary antibodies were purchased from Cell Signaling (7076 and 7054). The antibodies were used according to manufacturer's instructions.

Elucidation of the different pathways involved in the pathophysiology of neurologic disorders will aid in increasing our understanding of seizure and psychiatric disorders, and emphasize the need to further explore the involvement of specific mitochondrial subunits. The results presented in this report show the ability of a natural drug, VLE, to prevent the downregulation of protein levels of complex I subunits. Although several studies reported that polyphenols improve brain function, their mechanism of action remains poorly understood. In fact, polyphenols were shown to be powerful antioxidants that can prevent reactive species formation by chelating trace elements involved in free radical production, scavenging reactive species, and protecting antioxidant defenses (13-15). Limitations of this study include the fact that we only examined a limited number of factors that are affected by or involved in the modulation of protein expression levels in neuroblastoma cells that were treated with VLE. Examining a broader range of factors may help further elucidate the role of VLE on

mitochondrial electron transfer chain protein levels. Using more specific techniques such as immunocytochemistry and mass spectrometry in future studies to verify the findings of the present study may aid in further elucidating the effect of VLE in preventing alterations in protein expression levels induced by oxidative stress.

In conclusion, the development of efficacious interventions for the treatment and management of neurologic disorders has been garnering much interest. The findings of this study showed that VLE provides a significant prevention of H_2O_2-induced downregulation in the protein levels of complex I. Although these findings must be validated by future studies, they provide clues for furthering our understanding of the mechanism of action of VLE, which may aid in the discovery of targets for the development of more specific pharmacological interventions for seizure and psychiatric disorders.

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3.3 CAPÍTULO 3

Suppression of oncoprotein Her-2 and DNA damage after treatment with Flavan-3-ol

Vitis labrusca *extract*

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Suppression of oncoprotein Her-2 and DNA damage after treatment with Flavan-3-ol *Vitis labrusca* extract

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Abstract

Hepatocellular carcinoma and breast cancer are the most prevalent cancers in the world with high morbidity and mortality. Although there are effective drugs for treating advanced stages of liver and breast cancers, the prognosis for patients with liver cancer remains poor, and patients with breast cancer show considerable mortality. Therefore, it is crucial to explore new therapeutic agents for the inhibition of carcinogenesis. This study examined the anti-carcinogenic effect of *Vitis labrusca* seed extract (VLE), which is a component of winery waste, on liver (HepG2) and breast cancers (MCF-7) cells. The results found in this study demonstrated VLE-induced DNA damage in liver and breast cancer cells. VLE treatment in both cell lines was accompanied by high NO production and upregulation of p53. A significant decreased in total PARP expression was also found in HepG2 cells. In the MCF-7 cell line, VLE treatment increased the expression of BAX and AIF, and decreased total PARP expression. Surprisingly, VLE suppressed Her-2 expression in HepG2 cells and caused a subtle, but significant downregulation of Her-2 in MCF-7 cells. The possible anti-

carcinogenic effect of VLE reported in this study suggests the potential of this extract to be used for the development of novel therapeutic agents for the treatment of different kinds of cancers.

1. Introduction

Natural product-based drugs are receiving increasing attention in recent years. The increasing popularity of the use of phytotherapy-based medicines as preventive medicines or for health management has also driven research in these areas [1]. *Vitis labrusca* is one of the main grape species found in the Americas, and it is widely used to produce wines and grape juices [2]. Little is known about the phytochemical composition of *V. labrusca cv.* 'Bordo or Yves', and its biological activities. Every year, global wine production generates approximately 19.5 million tons of waste, which is used as fertilizer or is simply discarded in the environment [3]. Although some polyphenols are transferred from the grapes to the wine during vinification, the seed wastes are excellent sources of phenolic compounds [3-5].

Polyphenols found in grapes are divided into two groups: (a) phenolic acids and related compounds, and (b) flavonoids. Cinnamic and benzoic acids are major constituents of phenolic acids in grapes. Flavonoids include colorless flavan-3-ol compounds such as catechin, epicatechin, epigallocatechin and their polymers [6]. It has been shown that flavan-3-ols present many biological effects including the chelation of transition metals [7], as well as the modulation of proteins [5]. Because polyphenols exhibit potent free radical-scavenging properties, they have beneficial health effects by acting as antioxidants. On the other hand, some procyanidins are able to modulate cell functionality by affecting intracellular signaling cascades and gene expression [8]. Also, these compounds have been shown to have cytotoxic effects on tumor cells without having adverse effects on normal cells [9].

Worldwide, hepatocellular carcinoma is the fifth most common cancer in men and the eighth most common in women [10]. Moreover, breast cancer comprises around 16% of all female cancers [11]. The incidence/mortality of these two cancers have increased in recent years [12, 13]. Despite advances in diagnosis and treatments, the prognosis for patients with liver cancer remains poor [13] and patients with breast cancer show considerable mortality [14]. For this, it is crucial to research new therapeutic agents for the inhibition of carcinogenesis.

In this study, an extract made from the seeds of *V. labrusca* obtained from winery wastes was used to assess its effectiveness against liver (HepG2) and breast cancer (MCF-7) cells. The results showed that VLE was cytotoxic to both cell lines and presented distinct pathways to induce changes in cell dynamics through the activation of p53, Bax or AIF. The extract decreased the expression of two main proteins total PARP and Her-2, which are responsible for cellular proliferation and cell death.

2. Materials and methods

2.1. Chemicals

All chemicals used were of analytic grade. Complete Dulbecco's Modified Eagle's Medium (DMEM), penicillin, streptomycin, trypsin-EDTA and fetal bovine serum were purchased from Gibco. Thiazolyl blue tetrazolium bromide (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)-(MTT), Anti-PARP antibody produced in rabbit, Monoclonal anti-β-actin-peroxidase antibody produced in mouse, Low-melting agarose and Acrylamide kit were purchased from Sigma-Aldrich. Bax antibody (Human Specific) was purchased from Cell Signaling Technology. C-ERBB-2/HER-2 SPM495 antibody was obtained from Spring Bioscience and p53 (3H2821): sc-56179 mouse monoclonal antibody was purchased from Santa Cruz Biotechnology. Other reagents and solvents were obtained from Merck.

2.2. Vitis labrusca extract

Seeds from winery wastes of *V. labrusca* (cv. Bordo or Yves) were removed from the vinification tanks five days after beginning of fermentation. Seeds were manually separated from the rest of the winery wastes, the extracts were obtained using 5 g of seeds/100 mL of distilled water, under reflux (100 °C) for 30 min, filtered through a 0.45 µm pore (SFGS 047LS, Millipore Corp.) and freeze-dried (Edward freeze dryer). The extract was solubilized in DMEM medium immediately before use. The major compounds of the extract characterized by HPLC are listed in Figure 1.

2.3. Cell cultures

Cancer cell lines HepG2 (HB-8065) and MFC-7 (HTB-22), were cultured in DMEM medium with 1.85 g/l sodium bicarbonate, supplemented with 10% fetal bovine serum and 1% of penicillin/streptomycin. The both cell lines were purchased from ATCC, seeded in culture flasks, and maintained in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C for 3–6 days before experimentation.

2.4. Cellular viability assay (MTT assay)

Cell proliferation and cytotoxic effects were assessed using the MTT assay [15] in 96-well tissue culture plates. For cell proliferation, $1x10^4$ cells, per well, were used in different times (24, 48 and 72 h) to examine the growth behavior of the both cell lines using only the supplemented culture medium. The cytotoxic effects of VLE were analyzed in different concentrations (0.001 to 7 mg/mL) and different times (24, 48 and 72 h) on $1x10^4$ cells, per well. Briefly, after each cell cultivation experiment, 1:3 - 1mg/ml MTT solution was added per well and the cells were cultured for another 2 h. When the incubation was ended, the supernatant fluid was discarded. The purple-colored precipitates were dissolved within 150 µl DMSO per well, agitated for 10 min and the optical density (OD) of the resultant solution was

assayed with a VICTORX3 microplate reader (PerkinElmer) at 517 nm. The increase or reduction in viability of cells in each well was expressed as the percentage compared to non-VLE treated control cells.

2.5. Nitric oxide measurement

Nitrate, a stable end product of nitric oxide (NO) was determined in culture supernatants on 24, 48 and 72 h after VLE treatment by a method based on the Griess reaction [16]. Briefly, 100 µl of supernatant was plated in 96-well plates in triplicate and incubated with 100 µl of Griess reagent (1% sulfanilamide/0.1% N-(1-naphthyl)ethylenediamine dihydrochloride) in 3% H_3PO_4 at room temperature for 10 min. The optical density was measured at 535 nm using a VICTORX3 microplate reader (PerkinElmer). A standard curve was performed using sodium nitroprusside (SNP) for calibration. Protein concentration was determined by the Bradford method using bovine serum albumin as standard.

2.6. Genotoxicity assay

Single cell gel electrophoresis or comet assay was performed to assess potential genotoxic effects of VLE on HepG2 and MCF-7 cells. For this assay, $2.5x10^5$ cells were seeded in 6-well tissue plates and were treated with 0.1 and 1 mg/mL of the extract for 72 h. After, cells were harvested with trypsin-EDTA solution (0.25%) and resuspended in 500 µL of culture medium. Slides were prepared by mixing 20 µl cells suspension and 80 µl low melting point agarose (0.75%). The mixture was poured onto a frosted microscope slide coated with normal melting point agarose (1%). After solidification, the coverslip was removed and the slides were placed in lysis solution (2.5 M NaCl, 100 mM EDTA and 10 mM Tris, pH = 10.0-10.5, with freshly added 1 ml Triton X-100 and 10% DMSO) for 24 h. Subsequently, the slides were incubated in freshly made alkaline buffer (300 mM NaOH and 1 mM EDTA, pH = 12.6) for 20 min. The DNA was electrophoresed for 20 min at 25 V (0.9

V/cm) and 300 mA, and the buffer was neutralized with 0.4 M Tris (pH = 7.5). Finally, DNA was stained with silver nitrate, and the slides were coded for blind analysis. Images of 100 randomly selected cells (of four replicated slides) were analyzed from each sample. The damages were visually scored according to tail size into five classes, from no tail (0) to maximal (4) long tail, resulting in a single DNA damage score for each treatment, consequently, for each cell line. Therefore, a group damage index (DI) could range from 0 (all cells with no tail, 100 cells ×0) to 400 (all cells with maximally long tails, 100 cells ×4) [17].

2.7. Immunoblotting analysis

After 72 h of treatment with VLE, the cells were harvest and were mixed with Laemmli sample buffer, denatured by boiling (100 °C) for 5 min. Three thousand cells/µL (20µL) were loaded and the proteins were separated on 7.5-12.5% SDS-PAGE gels. After, the proteins were electro-transferred onto nitrocellulose membranes (Amersham Hybond™-C Extra). The membranes were blocked with 5% nonfat dry milk and were stained with primary antibodies overnight at 4 °C (p53, Bax, AIF, Her-2, total PARP and β-actin, as loading control). Then, the membranes were washed 3 times with PBST (PBS-buffered saline containing 0.1% Tween-20) to remove unbound primary antibody, stained with secondary antibodies anti-Rabbit IgG conjugated–peroxidase antibody or Mouse Ig, horseradish peroxidase-linked whole antibody for 1 h at room temperature. The wash procedure was redone followed by chemiluminescence protocol (Amersham Bioscience). All antibodies were used at 1:1000 dilutions except for PARP and Anti-Rabbit IgG conjugated–peroxidase antibody at 1:200 dilution and Mouse Ig, horseradish peroxidase-linked whole antibody at 1:5000. Protein band images were obtained using ImageScanner™ III (Ge Healthcare) and pairwise comparisons of the protein bands on the immunoblot were performed using Image-J 1.45 software.

2.8. Statistical analysis

All measurements were performed at least in triplicate. Values were averaged and expressed along with the standard deviation (SD) values. Results were subjected to analysis of variance (one-way ANOVA) and Tukey´s post-hoc test using SPSS 19.0 software (SPSS Inc., Chicago, IL).

3. Results

3.1 VLE phenolic characterization

The main phenolic compounds in VLE quantified by HPLC were catechin, followed by epicatechin, epigallocatechin, and procyanidins (Figure 1).

3.2. HepG2 and MCF-7 cell proliferation

The proliferation of human hepatocarcinoma cell line, HepG2, and breast cancer cell line, MCF-7, were analyzed. HepG2 cells proliferated faster when compared to MCF-7 cells (Figure 2) after 24, 48 and 72 h of incubation.

3.3. VLE treatment induces HepG2 and MCF-7 cell death

In the present study, we analyzed the sensitivity of HepG2 and MCF-7 cell lines to VLE treatment. Different concentrations and duration of treatment were used to evaluate the cytotoxic effects of VLE on these cell lines. Lower concentrations (0.001 to 0.1 mg/mL) did not show cytotoxicity against both cell lines in 24 and 48h, but had a cytotoxic effect in 72 hours. Following 24h (Figure 3A) of treatment, it was possible to observe an increase of mortality in HepG2 cells from 2mg/mL, and 3mg/mL to 7 mg/mL of VLE for both cell lines. In 48 h (Figure 3B), increased mortality was observed in concentrations greater then 1mg/mL. Interestingly, cytotoxicity increased in a dose-dependent manner up to 3mg/mL of VLE, where a plateau was reached (Figure 3A, B and C). At 72h of treatment, the cytotoxicity

profile of VLE for both cell lines showed a different pattern (Figure 3C). More specifically, starting from 0.1mg/mL of VLE, HepG2 cells showed heightened sensitivity to the extract compared to MCF-7 cells. After these analyses, 0.1mg/mL and 1mg/mL of VLE for 72h of treatment were chosen to verify the mechanism of VLE-induced cell in both cell lines.

3.4. VLE treatment induces NO production in HepG2 and MCF-7

NO measurements showed that 1 mg/mL of VLE induced a 3.9 (24h), 4.7 (48h), and 4.8 (72h) fold increase in NO production compared to control in HepG2 cells (Table 1). For MCF-7, 1 mg/mL of VLE induced a 2.1 (24h), 2.2 (48h) and 2.4 (72h) fold increase in NO compared to control (Table 1).

3.5. VLE treatment induces HepG2 and MCF-7 DNA damages

The genotoxic effect of VLE was assayed, for 72 h of treatment. DNA damage was evaluated first, by the overall score on the DNA damage index (DI), and second, by the frequency of cells in each class of DNA damage. It was found that DI for 0.1 and 1mg/mL of VLE treatment on HepG2 was approximately 25 and 50 times higher than the control, respectively (Figure 4A). MCF-7 did not present a genotoxic effect for 0.1 mg/mL treatment, but had a significant increase (0.7 fold) on DI for 1 mg/mL treatment (Figure 4A). For HepG2, majority of the cells were found in classes one and two of DNA damage for 0.1mg/mL of VLE. For 1mg/mL of VLE, majority of the cells were found in classes 2 and 3, demonstrating an increase in genotoxicity (Figure 4B). MCF-7 presented a different profile for DNA damage frequency when compared to HepG2. The distribution of cells for 0.1 mg/mL of treatment did not show a significant difference when compared to control, while for 1 mg/mL of treatment, the cells were found in classes one and two, demonstrating a low level of genotoxicity.

3.6. VLE modulates the expression of apoptotic proteins in HepG2 and MCF-7 cells

To investigate the involvement of apoptotic signaling molecules following VLE treatment, we examined the expression levels of p53, Bax, and AIF through immunoblotting. Our results showed that the expression of apoptotic signaling proteins was modulated by VLE. In the highest concentration of VLE, a significant increase in p53 expression was found, while Bax was found to be unchanged in HepG2 cells. For MCF-7, treatment with VLE produced a marked increase in Bax and AIF, while p53 showed a modest, but significant increase (Figure 5).

3.7. VLE reduces Her-2 and PARP expression in HepG2 and MCF-7 cells

Oncoprotein Her-2 and total PARP expression were also analyzed by immunoblotting. Intriguingly, Her-2 and PARP were significantly decreased for HepG2 cells following treatment with VLE in both concentrations. Moreover, on MCF-7 cells, expression of Her-2 and PARP showed a slight but significant decrease (Figure 6).

4. Discussion

Over the years, there has been a global interest in flavan-3-ol grape seed extracts as a dietary supplement because of its various health benefits including anti-mutagenesis and anti-carcinogenesis [18, 19]. It was also reported by the U.S. Environmental Protection Agency´s Health Effects Test Guidelines and the Toxic Substances Control Act that extracts from grape seeds are safe for human consumption [20]. Furthermore, it has been linked to cancer prevention/therapy and exhibits cytotoxicity to cancer cells but not to normal cells [9]. In addition, several studies were reported using different extracts from grapes or grapes seeds showing that grape polyphenols could be used in standard chemotherapeutic regimens for various forms of cancers [5, 21-23]. However, the molecular mechanisms of its anti-carcinogenic effects in liver and breast cancer cells are not clearly understood.

A major finding of the present study was that HepG2 is more vulnerable to VLE-induced damage than MCF-7. This may be explained by the fact that the cell cycle of HepG2 is faster than MCF-7. More specifically, because of its slower cell cycle, MCF-7 may be less vulnerable to the accumulation of VLE-induced cell injury. It has been suggested that flavan-3-ol exerts its anti-cancer activity due to its pyrogallol-type structure [22, 24]. In fact, previous reports have reported that epigallocatechin gallate induces apoptosis in colon cancer cells [22, 25]. Importantly, this effect was found to be very weak for catechin and epicatechin, which lack a galloyl group, suggesting the presence of a structure-function relationship in the ability to induce apoptosis [22]. Therefore, it is likely that the pyrogallol-type structure in the B-ring of flavan-3-ol contributes more to its apoptosis-inducing activity [22] (Figure 1).

VLE has been shown to have anti-proliferative and cytotoxic activity *in vitro* in liver and breast cancer cells (Figure 3) and increased the expression of markers of apoptosis through mitochondrial intrinsic pathway for MCF-7 cells (Figure 5A). Inhibition of apoptosis is considered to be one of the possible mechanisms of tumor development, and many chemopreventive agents have been shown to act through the induction of apoptosis to inhibit or block the carcinogenic process [18].

Uncontrolled cell growth and resistance to apoptosis are major defects in neoplasia. Development of approaches that promote apoptotic machinery within cancer cells could be effective against their proliferation and invasive potential [26]. In this study, VLE produced significant changes in HepG2 and MCF-7 cells, which included growth inhibition (<1mg/mL), cytotoxic effects (>1mg/mL), induction of markers of DNA damage, alterations in cell signaling pathways, and downregulation of Her-2 and total PARP expression.

The anti-cancer mechanism of different polyphenolic compounds can be attributed to their pro-oxidant and DNA damaging effects [27]. VLE increased the production of NO in both cell lines (Table 1), which through the nitrosoperoxycarbonate pathway can cause DNA single-strand breaks, cause damage to DNA bases (Figure 4A and B) [28], and produce

cytotoxicity at high concentrations [29]. Moreover, NO forms peroxynitrite (ONOO-) and nitrous anhydride (N_2O_3) through its reactions with superoxide and oxygen. These reactive species can cause protein and lipid-peroxidation, which creates secondary metabolites [30] that can lead to cell death [29]. Additionally, NO can also stimulate p53 accumulation, inducing programmed cell death through the process of apoptosis to remove cells at are unable to repair damaged DNA [29].

HepG2 and MCF-7 cells express wild-type p53, which is a tumor-suppressing protein. p53, which can act as a tumor suppressor, has been shown to be increased in response to DNA damage [31]. In turn, p53 orchestrates a global transcriptional response that either counters cell proliferation/senescence or induces apoptosis [5, 31]. Liver and breast cancer cells treated with VLE with increasing doses exhibited a dose-dependent increase in p53 expression (Figure 3 and 5). However, the magnitude of increase in the expression of p53 with 1 mg/mL of VLE was greater in HepG2 cells than in MCF-7 cells, demonstrating that HepG2 cells could have a higher sensitivity to VLE treatment.

The Bax protein is considered to be one of the primary targets of p53 and controls cell death through its participation in the disruption of mitochondria [32]. VLE induced an increase in the expression of Bax protein in MCF-7 cells. Bax normally acts as a sensor of cellular damage and stress. In response to significant damage or stress, Bax relocates to the mitochondrial surface and disrupts the normal functioning of the anti-apoptotic Bcl-2 protein [23, 33]. Bax can produce transmembrane pores across the outer mitochondrial membrane, which leads to loss of membrane potential [33] and efflux of cytochrome c and the AIF.

The increased DNA fragmentation found in MCF-7 cells in the present study, following mitochondrial outer membrane permeabilization, AIF can be released into the cytosol and translocate to the nucleus where they bind to DNA to facilitate fragmentation and nuclear condensation [34]. VLE-induced increase in Bax levels would facilitate AIF release

[35], leading to cell death for MCF-7. On the other hand, VLE did not change levels of Bax and AIF proteins in HepG2.

VLE significantly downregulated total PARP expression in HepG2. Engelbrech et al. [36] reported a similar finding using colon cancer cell lines. This protein, which is an important marker of apoptosis, takes part in the base excision repair (BER) pathway for DNA single strand breaks. This makes PARP an interesting target for cancer therapy. Inhibiting PARP may be sufficient to cause tumor cell death [37]. Although certain tumors with DNA repair defects are very sensitive to PARP inhibition, only a few PARP-inhibitors have been discovered until now [37]. Some inhibitors of PARP are currently in clinical trials as cancer therapeutics, yet the specificity of these compounds is still unknown [38].

Another important target for cancer therapy is Her-2, which is a transmembrane protein with tyrosine kinase activity [39]. This protein is responsible for the regulation of cellular proliferation, differentiation, motility and apoptosis [40]. While Her-2 overexpression only occurs in 2.42% of liver cancer patients, 20 – 25% of breast cancer patients show Her-2 positive status [41]. Importantly, Her-2 overexpression is correlated with aggressive tumour behaviour, poor prognosis [42], and resistance to drug treatment [42]. Therefore, there is an urgent need for the development of therapeutic interventions that specifically target Her-2 in breast cancer.

In our study, VLE suppressed Her-2 expression in HepG2 cells and MCF-7 cells, suggesting that this extract could be used for the development of therapeutic reagents against Her-2 positive cancers. Moreover, VLE presented an anti-carcinogenic effect that could be attributed to the induction of DNA damage and cell death. Future studies can further explore the potential of this extract as a therapeutic agent by using *in vivo* models, and also by testing the effect of VLE against Her-2 in combination with established therapeutic agents, such as Trastuzmab. This is especially of interest since Trastuzumab has a limited response rate

ranging from 15% to 40% [43], indicating a need for the development of adjuvants to increase its efficacy.

Lastly, this study only examined a limited number of factors that are affected by or involved in VLE's anti-carcinogenic effects. Examining a broader range of factors, such as changes in the expression patterns of specific proteins involved in the signaling pathway of PARP and Her-2, alterations in DNA, and the effect of combining VLE with other chemotherapeutic drugs will help to further elucidate the mechanism of the extract. Importantly, this will allow for the validation of VLE's potential to be used as an adjuvant in chemotherapeutic regimens.

4. Conclusion

The findings of our study suggest that VLE presented an anti-carcinogenic effect that can be attributed to the induction of DNA damage and cell death. VLE induced an increase on NO production, leading to oxidative DNA breakage. On HepG2 cells, VLE stimulated an increase in p53 levels, which downregulated total PARP. In MCF-7, p53 activated Bax, which released AIF, and downregulated total PARP. Importantly, VLE caused suppression of the oncoprotein, Her-2, in HepG2 and MCF-7 cells. Together, these results suggest that VLE regulates important anti-carcinogenic pathways that synergistically could induce liver and breast cancer cell death. This demonstrates the potential of compounds present in winery wastes seeds to be developed as therapeutic agents against different types of cancers.

Conflict of interest

The authors do not have any conflict of interest to disclose.

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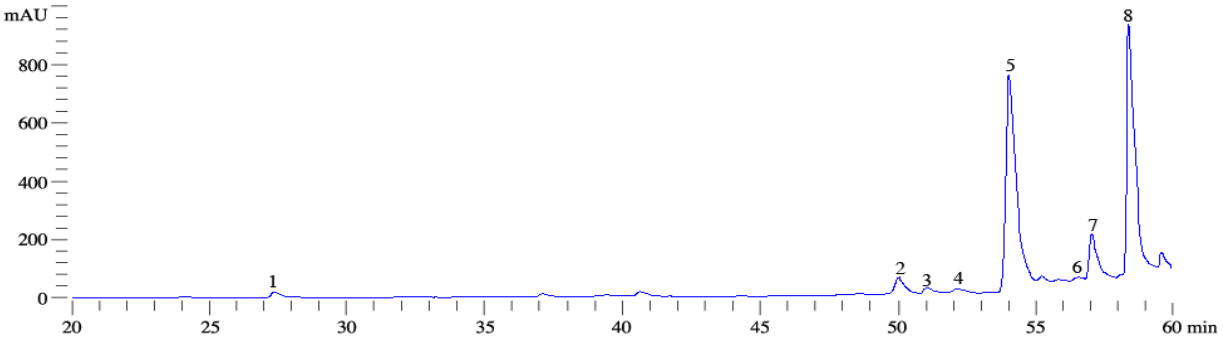
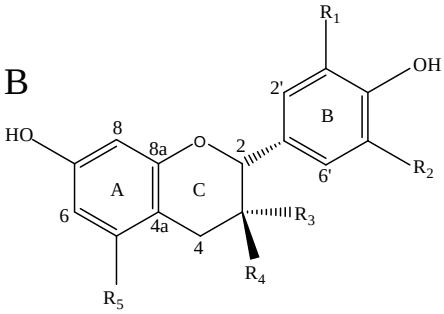
A

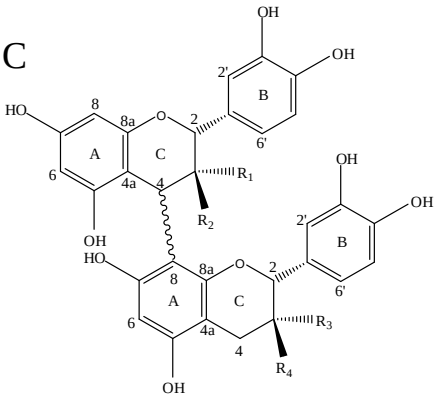


B



Flavan-3-ol monomers of VLE	R_1	R_2	R_3	R_4	R_5
(+)-catechin	H	OH	H	OH	OH
(-)-epicatechin	H	OH	OH	H	OH
(-)-epigallocatechin	OH	OH	OH	H	OH

C



Flavan-3-ol dimers of VLE	R_1	R_2	R_3	R_4
B1	OH	H	H	OH
B2	OH	H	OH	H
B3	H	OH	H	OH
B4	H	OH	OH	H

D

Vitis labrusca extract	Total phenolic content	Major compounds			
		Catechin	Epicatechin	Epigallocatechin	Procyanidins B1, B2, B3 and B4
	744.89±3.13	169.26±0.92	168.86±2.82	8.96±0.05	22.42±1.01

Fig. 1. Characterization of the VLE major compounds. (**A**) HPLC of VLE recorded at 204 nm. (1) gallic acid, (2) procyanidin B1, (3) epigallocatechin, (4) procyanidin B3, (5) catechin, (6) procyanidin B4, (7) procyanidin B2, (8) epicatechin. (**B**) Flavan-3-ol monomers and (**C**), dimers found in VLE. (**D**) Total polyphenol content (mg/L of catechin) and major compounds (mg/L) in the VLE are represent as average values ± S.D. (data adapted from Scola et al., 2010).

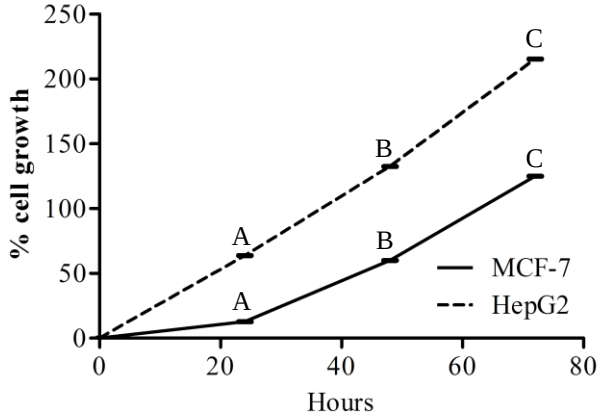


Fig. 2. Cellular growth of HepG2 and MCF-7 cells on 24 h (A), 48 h (B) and 72 h (C) of treatment measured by MTT assay. % cell growth = [(number of cells at time of observation / original number of cells) - 1] * 100. Therefore, 50% cell growth is indicative of doubling time. Values were averaged and expressed along with the SD. values.

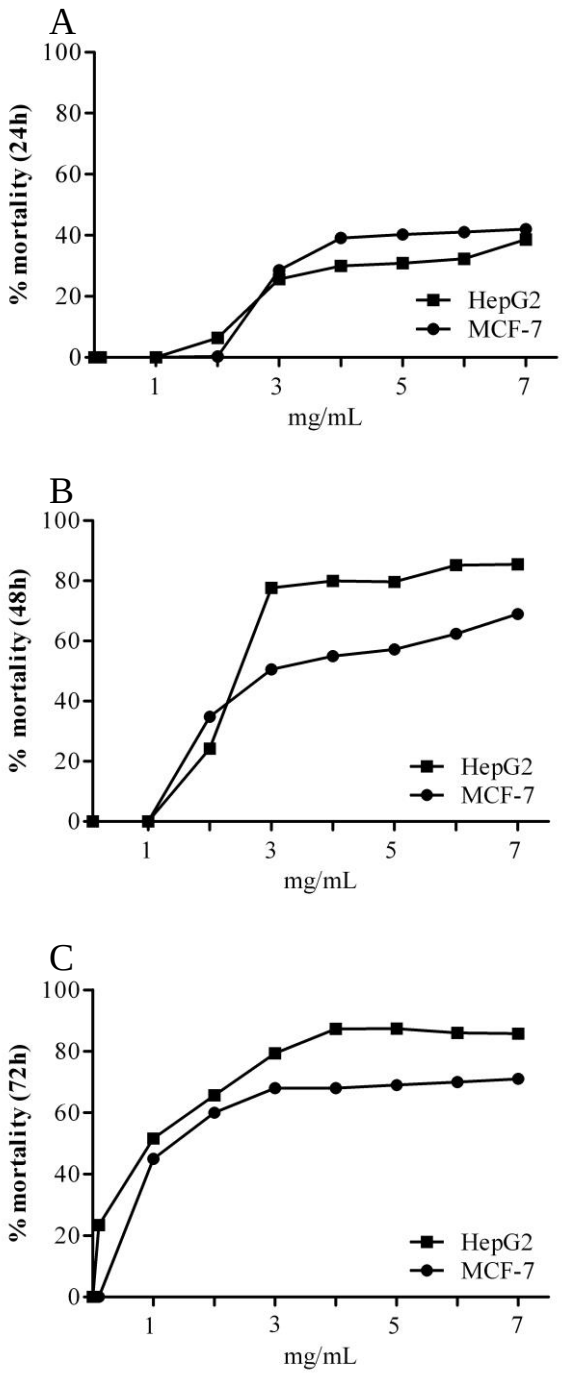
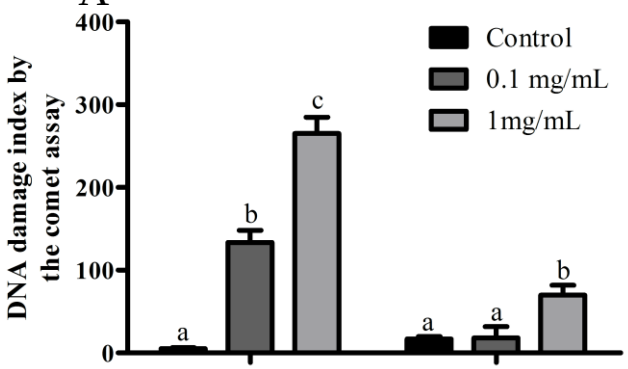


Fig. 3. Cytotoxic effects of VLE on HepG2 and MCF-7 cancer cells. Dose-dependent cytotoxic effect of VLE on HepG2 and MCF-7 cells for 24 h (A), 48 h (B) and 72 h (C) of treatment measured by MTT assay. % mortality = [(number of cells at time of observation / number of control cells) * 100]. Values were averaged and expressed with SD values.

A



B

	Undamaged cells	Damage class one	Damage class two	Damage class three	Damage class four
HepG2					
Control	94.50±1.29^{a*}	5.50±1.26^{b}	0.00±0.00^{c}	0.00±0.00^{c}	0.00±0.00^{c}
0.1 mg/mL	11.00±3.11^{a}	60.00±7.66^{b}	24.00±5.97^{c}	4.50±1.00^{a}	0.50±0.10^{a}
1 mg/mL	0.00±0.00^{a}	0.25±0.05^{a}	37.25±3.75^{b}	59.50±5.01^{c}	3.00±0.16^{a}
MCF-7					
Control	82.75±4.11^{a*}	12.75±3.20^{b}	4.50±0.00^{c}	0.00±0.00^{c}	0.00±0.00^{c}
0.1 mg/mL	86.50±5.51^{a}	13.00±4.76^{b}	0.50±0.05^{c}	0.00±0.00^{c}	0.00±0.00^{c}
1 mg/mL	45.25±5.85^{a}	42.50±4.43^{a}	9.50±0.90^{b}	2.75±0.56^{c}	0.00±0.00^{c}

Fig. 4. (**A**) DNA damage index by comet assay in HepG2 and MCF-7cells after 72 h treatment with VLE. Each cell was allocated to one of the five classes from 0 (undamaged) to 4 (maximally damage), according to the size and shape of the tail. The values obtained for each group could range from 0 (0 × 100) to 400 (4 × 100). (**B**) Frequency (%) of the different classes of DNA damage in control and VLE-treated groups for HepG2 and MCF-7. The cells were assessed visually and received scores from 0 (undamaged) to 4 (maximally damaged), according to the size and shape of the tail. Results represent average values ± S.D. *Superscript letters indicate significant differences according to ANOVA and Tukey´s post-hoc test ($p \leq 0.05$).

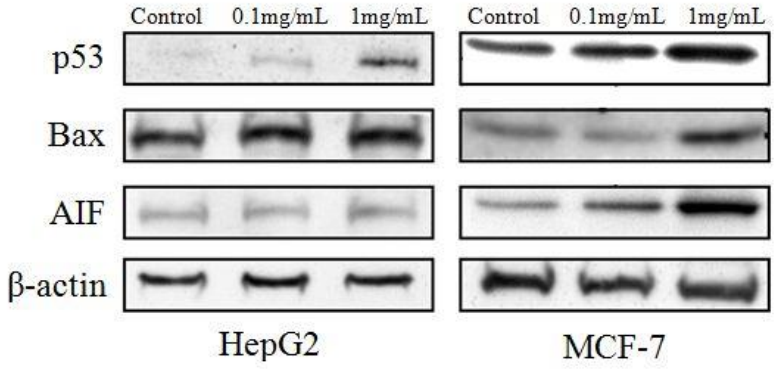


Fig. 5. Immunoblotting showing altered expression of apoptosis proteins, p53, Bax, and AIF, in HepG2 and breast MCF-7 cancer cells. Total cellular proteins of VLE-treated cells were separated and electro-transferred onto nitrocellulose membrane. β-actin serves as loading control.

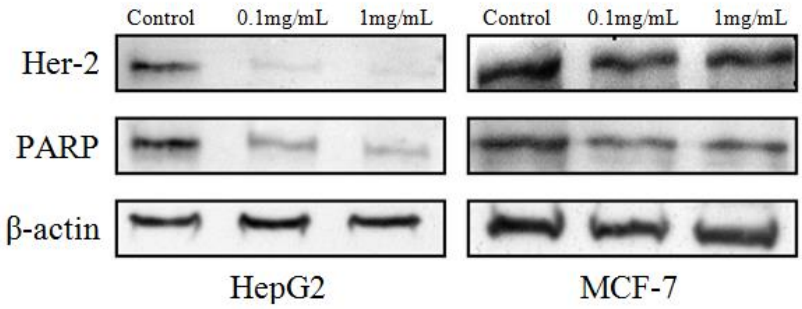


Fig. 6. Immunoblotting showing altered expression of proliferative proteins, Her-2 and PARP, in HepG2 and breast MCF-7 cancer cells. Total cellular proteins of VLE-treated cells were separated and electro-transferred onto nitrocellulose membrane. β-actin serves as loading control.

Table 1. Quantification of nitric oxide in control and VLE-treated groups for HepG2 and MCF-7 cells

	Treatment time (h)		
	24	48	72
HepG2			
Control	2.56±0.02[a*]	2.56±0.01[a]	2.72±0.01[a]
0.1 mg/mL	2.42±0.01[a]	2.81±0.03[a]	2.93±0.01[a]
1 mg/mL	10.00±0.01[b]	12.22±0.01[b]	13.10±0.01[b]
MCF-7			
Control	16.59±0.01[a*]	18.06±0.01[a]	19.54±0.01[a]
0.1 mg/mL	16.72±0.01[a]	18.39±0.01[a]	21.74±0.01[b]
1 mg/mL	35.83±0.01[b]	40.17±0.01[b]	48.31±0.01[c]

Results represent average values ± S.D and are expressed as nitric oxide content (mg% SNP/mg of protein). [*]Superscript letters indicate significant differences according to ANOVA and Tukey´s post-hoc test ($p \leq 0.05$).

DISCUSSÃO GERAL

4. DISCUSSÃO GERAL

Mesmo com o pouco interesse das grandes indústrias farmacêuticas no uso de fitoterápicos, percebe-se um aumento no número de patentes descrevendo novas utilidades para compostos naturais. Diversas moléculas, pertencentes às mais variadas classes químicas, tem ganho destaque na medicina, contribuindo para o tratamento de doenças do sistema imune, doenças neurológicas e do câncer (Ferguson, 2001, Aron & Kennedy, 2008, Butler, 2008, Ferguson & Philpott, 2008, Newman & Cragg, 2009, Nicolaou *et al.*, 2009, Barreiro & Bolzani, 2009, Monagas *et al.*, 2010).

Anualmente, a produção de vinhos no mundo ultrapassa os 150 milhões de hectolitros (OIV, 2008), gerando grande quantidade de resíduos (Torres *et al.*, 2002). Dados prévios (Scola *et al.*, 2010, Scola *et al.*, 2011) mostraram que estes resíduos (*V. vinifera* - Cabernet Sauvignon e Merlot, e de *V. labrusca* - Isabel e Bordo) são ricos em compostos fenólicos e apresentaram significativa atividade antioxidante *in vitro* e *in vivo* (Scola *et al.*, 2010) e atividade anti-inflamatória *in vivo* (Scola *et al.*, 2011)(Anexos 1 e 2). Destes resíduos, o da variedade Bordo foi o que apresentou maior teor de polifenóis e maior atividade antioxidante, justificando, assim, a continuidade dos estudos acerca de seus efeitos biológicos.

O capítulo 1 apresenta os efeitos do extrato de *V. labrusca* na prevenção/redução de danos oxidativos induzidos por crises convulsivas em ratos Wistar. Para tanto, diferentes concentrações do extrato foram administradas (10, 50 e 100mg/kg) aos ratos, previamente a administração intraperitoneal de pentilenotetrazol (60mg/kg; PTZ). O PTZ é, reconhecidamente, um agente convulsivo que altera as concentrações dos diferentes

neurotransmissores do cérebro, ocasionando o aumento do fluxo sanguíneo, o aumento da permeabilidade da barreira hematoencefálica e a perda da regulação das funções cerebrais (Obay *et al.*, 2008, Luttjohann *et al.*, 2009, Szyndler *et al.*, 2010). Além disso, o PTZ intensifica os efeitos do neurotransmissor inibitório glutamato, ocasionando um aumento na produção de espécies reativas (Costello & Delanty, 2004, Obay *et al.*, 2008, Luttjohann *et al.*, 2009, Szyndler *et al.*, 2010), associada à disfunção mitocondrial (Costello & Delanty, 2004, Chuang, 2010, Schaffer & Halliwell, 2012). Por sua vez, o estresse oxidativo apresenta importante efeito na dinâmica celular, podendo levar à morte neuronal (Costello & Delanty, 2004, Sharma *et al.*, 2010).

Os resultados mostraram que o pré-tratamento com o extrato de *V. labrusca* foi capaz de prevenir os danos oxidativos aos lipídeos e proteínas induzidos pela convulsão, tanto no SNC quanto no fígado dos ratos. A dose de extrato utilizada foi cerca de 500 vezes menor do que a dose de epigalocatequina-3-galato (polifenol presente também no chá verde) empregada para obtenção de efeitos similares no SNC de ratos *Sprague-Dawley* (Xie *et al.*, 2012).

Embora o tratamento com o extrato de *V. labrusca* não tenha evitado as convulsões induzidas pelo PTZ, observou-se uma tendência no aumento do tempo de latência para o início da convulsão (dados não mostrados). O tratamento com as diferentes concentrações do extrato evitou a mortalidade induzida pelo PTZ. Sabe-se que os polifenóis podem alterar as funções cerebrais (modificando diretamente a atividade celular neuronal), o fluxo sanguíneo e modular a seletividade da barreira hematoencefálica (Schaffer & Halliwell, 2012), o que poderia explicar, ao menos em parte, a redução da mortalidade observada neste trabalho.

Quando testado em células de neuroblastoma humano (SH-SY5Y), o extrato de *V. labrusca* minimizou a redução na expressão das proteínas NDUFV2, NDUFS8 e NDUFS7, do complexo I da cadeia transportadora de elétrons, induzida pelo H_2O_2 (Capítulo 2). Scola *et al*. (2013), revisando as alterações gênicas na cadeia transportadora de elétrons de pacientes com diagnóstico de transtorno de humor bipolar, observou que 8 genes relacionados à atividade e

estrutura do complexo I apresentavam seus níveis de expressão menores quando comparados a pacientes sem diagnóstico de transtorno bipolar. Destes, os genes NDUFV1, NDUFV2, NDUFS1, NDUFS8 e NDUFS7 são relacionados com a atividade do complexo I (Janssen *et al.*, 2006) e seu funcionamento pode ser modulado através de modificações induzidas pelo estresse oxidativo (Andreazza *et al.*, 2010, Gigante *et al.*, 2010).

As subunidades NDUFV1 e NDUFV2 são responsáveis pela transferência dos elétrons da nicotinamida adenina dinucleotídeo para os demais grupos ferro-enxofre pertencentes ao complexo I, onde as subunidades NDUFS1 e NDUFS8 atuam apenas como transferidores dos elétrons para a subunidade NDUFS7, esta responsável pela redução da ubiquinona a ubiquinol (Anexo 3). Recentemente, pesquisadores da área de psiquiatria vêm discutindo a utilização de compostos antioxidantes na prevenção e manutenção do balanço redox nas desordens psiquiátricas (Berk *et al.*, 2008, Dodd *et al.*, 2008, Dean *et al.*, 2011).

Em conjunto, estes resultados mostram que o extrato de resíduos de vinificação de *V. labrusca* previne os danos oxidativos aos lipídeos e proteínas, evitando a morte neuronal sem causar genotoxicidade ou mutagenicidade.

Além dos efeitos relacionados ao SNC, o extrato de *V. labrusca* também se mostrou hepatoprotetor, modulando a atividade das enzimas SOD (diminuindo) e CAT (aumentando) e prevenindo os danos oxidativos aos lipídeos e as proteínas em ratos Wistar.

Sabendo que alguns polifenóis podem apresentar efeito antitumoral (Dinicola *et al.*, 2010), testou-se os efeitos do extrato de *V. labrusca* em células de câncer de fígado (HepG2) e de mama (MCF-7). Em concentrações mais baixas (até 1 mg/mL) o extrato mostrou efeito citostático nestas linhagens; já em concentrações acima de 1 mg/mL, observou-se um efeito citotóxico, com redução do crescimento celular de cerca de 80%. Este efeito foi acompanhado pelo aumento da produção de óxido nítrico, dano ao DNA, ativação do maquinário apoptótico e a regulação de importantes proteínas (Her-2 e PARP) relacionadas à proliferação e reparo

celular. Um resumo das principais atividades biológicas relacionadas à utilização do extrato de *V. labrusca* pode ser observado na Figura 3.

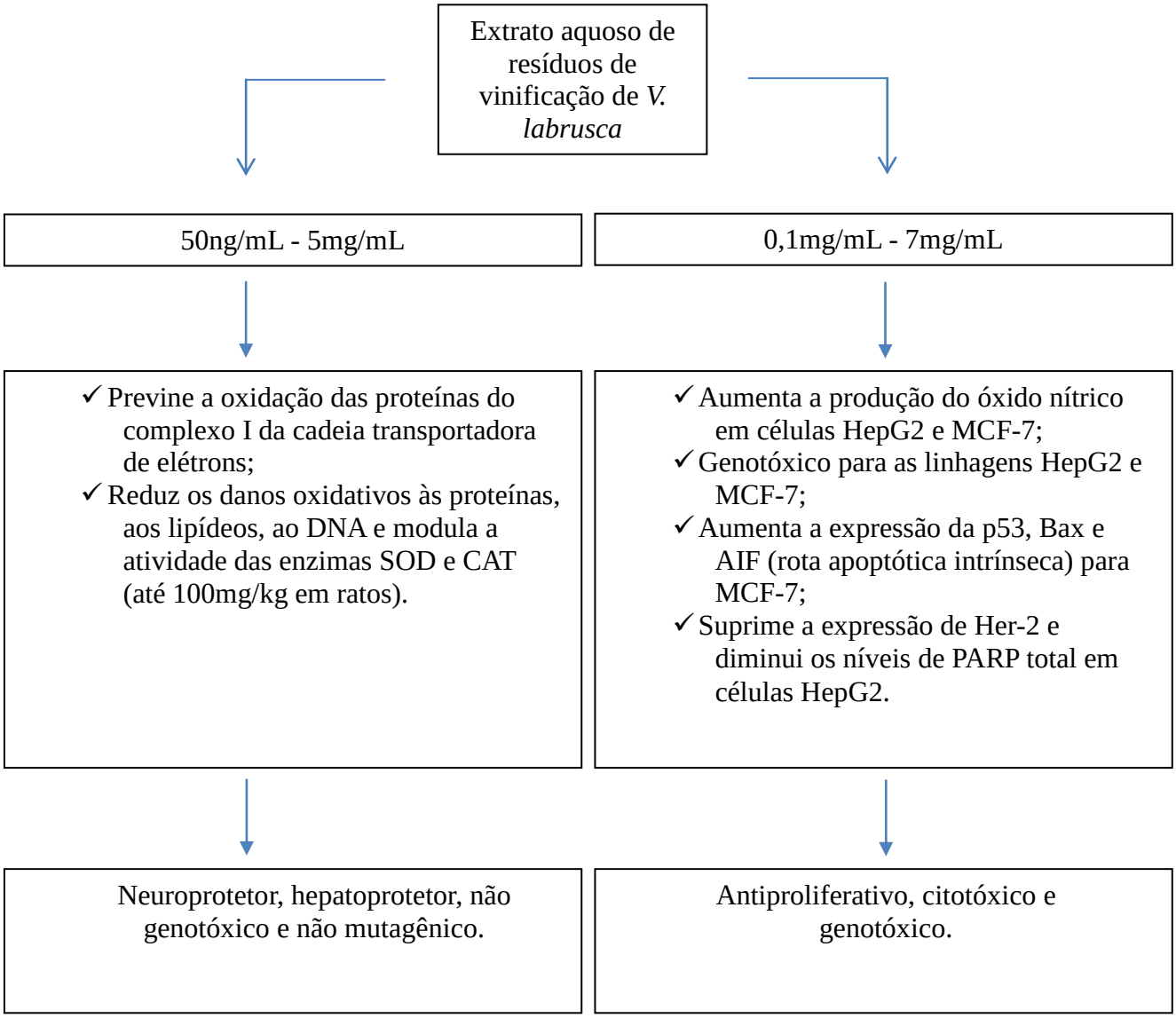


Figura 3. Resumo das principais atividades do extrato de *V. labrusca*.

O tratamento com o extrato de resíduos de vinificação de *V. labrusca* aumentou os níveis de expressão da proteína p53 nas linhagens HepG2 e MCF-7. Para a linhagem MCF-7 o extrato aumentou os níveis de expressão do fator de indução de apoptose (AIF) e Bax, mostrando que o mecanismo de indução apoptótica foi ativado intrinsecamente, ou seja, através do dano mitocondrial (Green & Reed, 1998, Marzo *et al.*, 1998, Susin *et al.*, 1999,

Green & Kroemer, 2004, Dinicola *et al.*, 2010). Na linhagem de câncer de fígado, o tratamento com o extrato de *V. labrusca* apresentou menor aumento nos níveis de expressão da proteína Bax, indicando a necessidade de outros estudos a fim de verificar quais são as proteínas moduladas pelo extrato nesta linhagem celular.

O extrato de *V. labrusca* estimulou a produção de óxido nítrico nas linhagens de câncer de fígado e de mama. Sabe-se que o óxido nítrico pode reagir com o radical superóxido, oriundo das disfunções da cadeia de transporte de elétrons, e formar peroxinitrito e anidrido nítrico, responsáveis pela nitração das proteínas da cadeia transportadora de elétrons (el Ghissassi *et al.*, 1995, Ambs *et al.*, 1998). Mais ainda, as espécies reativas, quando em concentrações não fisiológicas, podem causar citotoxicidade e danos ao DNA (el Ghissassi *et al.*, 1995, Ambs *et al.*, 1998, Dinicola *et al.*, 2010).

Os resultados da medida de dano ao DNA induzido pelo extrato de *V. labrusca* mostraram um índice de danos maior na linhagem de câncer de fígado do que o observado para a linhagem de câncer de mama. Resultados semelhantes foram observados na avaliação da citotoxicidade induzida pelo extrato, onde a linhagem de câncer de fígado apresentou maior sensibilidade ao extrato. Mais ainda, e não menos importante, o extrato foi capaz de diminuir os níveis de expressão de PARP total e suprimir a Her-2 para linhagem tumoral de fígado, indicando uma maior sensibilidade desta linhagem ao tratamento com o extrato de *V. labrusca*.

Uma das estratégias que vem sendo desenvolvida é a procura de novos compostos terapêuticos específicos para modificações moleculares, como a inibição de proteínas oncogênicas e a ativação apoptótica, que são os alvos das novas terapias anticâncer (Boyle & Howell, 2010, Chen, 2011, Song *et al.*, 2012).

Em resumo, como apresentado na Figura 3 deste trabalho, os resultados apontam para a potencialidade dos resíduos de vinificação como fonte de compostos com atividade biológica. Além disso, este trabalho colabora com o entendimento do mecanismo de ação dos

polifenóis presentes no extrato. Pesquisas adicionais visando à elucidação do mecanismo destes compostos são importantes para confirmar estes resultados.

CONCLUSÕES

5. CONCLUSÕES

Os dados obtidos neste estudo permitem concluir que:

5.1 O extrato de *V. labrusca* minimiza os danos oxidativos aos lipídeos e proteínas induzidos pelo pentilenotetrazol, no córtex cerebral, hipocampo, cerebelo e fígado de ratos.

5.2 O tratamento com o extrato de *V. labrusca* não é genotóxico em linfócitos de ratos e não é mutagênico em células eucarióticas da levedura *S. cerevisiae*.

5.3 O extrato de *V. labrusca* apresenta significativa capacidade em prevenir a diminuição dos níveis de expressão das proteínas do complexo I da cadeia transportadora de elétrons em células de neuroblastoma humano (SH-SY5Y) tratadas com H_2O_2.

5.4 A administração do extrato de *V. labrusca* possui efeito citotóxico em células de câncer de fígado (HepG2) e mama (MCF-7).

5.5 O tratamento com o extrato de *V. labrusca* altera os níveis de expressão das proteínas p53, PARP e Her-2 em células de câncer de fígado (HepG2) e de mama (MCF-7) e altera os níveis de expressão das proteínas Bax e AIF em células MCF-7.

PERSPECTIVAS

6. PERSPECTIVAS

Para a continuidade deste trabalho, é possível:

6.1 Verificar se o aumento do tempo de administração do extrato de *V. labrusca* pode apresentar efeito anticonvulsivante em ratos.

6.2 Avaliar o efeito da combinação do extrato de *V. labrusca* com outras drogas anticonvulsivas, a fim de examinar os efeitos de um tratamento conjunto.

6.3 Investigar os níveis de expressão de outras proteínas relacionadas a apoptose e a PARP inativada em células de mamíferos tratadas com o extrato de *V. labrusca*.

6.4 Caracterizar quais são as alterações no DNA (níveis de metilação e hidroximetilação) induzidos pela administração do extrato de *V. labrusca*.

6.5 Avaliar possíveis modificações morfológicas e/ou moleculares no citoesqueleto das células tratadas com o extrato de *V. labrusca*.

6.6 Analisar o efeito da combinação do extrato de *V. labrusca* com outras drogas quimioterápicas, como a doxorubicina e a cisplatina em células tumorais.

6.7 Investigar o efeito biológico dos polifenóis encontrados no extrato de *V. labrusca*, isoladamente, a fim de identificar as moléculas mais ativas.

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ANEXOS

8. ANEXOS

8.1 Anexo 1

Flavan-3-ol Compounds from Wine Wastes with in Vitro and in Vivo Antioxidant Activity

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Article

Flavan-3-ol Compounds from Wine Wastes with *in Vitro* and *in Vivo* Antioxidant Activity

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Abstract: It has been suggested that the dietary intake of antioxidant supplements could be a useful strategy to reduce the incidence of diseases associated with oxidative stress. The aim of present work is to study the possibility to obtain compounds with antioxidant activity from wine wastes using water as solvent. Results have shown that it is possible to obtain flavan-3-ol compounds from wine wastes both from *V. vinifera* (cv. Cabernet Sauvignon and Merlot) and *V. labrusca* (cv. Bordo and Isabella) species. The main phenolic compounds found in the extracts were catechin and epicatechin, followed by procyanidin B3, procyanidin B1, procyanidin B2, gallic acid, epigallocatechin, and procyanidin B4. All flavan-3-ol extracts showed significant *in vitro* and *in vivo* activities. It was found that the extracts were able to prevent lipid and protein oxidative damage in the cerebral cortex, cerebellum and hippocampus tissues of rats. Although further studies are necessary, these flavan-3-ol extracts show potential to be used to reduce the incidence of degenerative diseases associated with oxidative stress.

Keywords: aqueous wine waste extracts; *V. vinifera*; *V. labrusca*; phenolic content; antioxidant

1. Introduction

The role of dietary supplements in the prevention of some diseases has received widespread attention [1]. It has been suggested that the dietary intake of antioxidant supplements could be a useful strategy to reduce the incidence of diseases associated with oxidative stress, such as cancer, atherosclerosis and neurodegenerative diseases [2]. Vegetables, fruits and their seeds are rich sources of antioxidant compounds, such as vitamins, beta-carotene and polyphenols. Among the fruits, grapes have high polyphenol content, and 60–70% of these compounds are found in grape seeds. Grape seed supplements, obtained from *Vitis vinifera* species using organic solvents, have been reported to have a broad spectrum of pharmacological effects, such as antioxidative, anti-inflammatory and antimicrobial activities, as well as cardioprotective, hepatoprotective and neuroprotective effects [3].

Among the polyphenols found in grapes, flavonoids are one of the most abundant groups [4], including colorless flavan-3-ol compounds such as catechin, epicatechin and their polymers [5]. It has been shown that flavan-3-ols possess many biological effects including the scavenging of free radicals, chelation of transition metals, as well as the modulation of some antioxidant enzymes [6].

Some of the polyphenols present in grapes are extracted into wine, but most remain in the vinification wastes (pomace, stems and seeds), which account for about 13% of the processed grape weight [7]. Every year, the worldwide wine production (around 260 million hL) generates about 19.5 million ton of waste, which usually end up being used as fertilizer or simply being discarded [8]. *Vitis vinifera* (cv. Cabernet Sauvignon and Merlot) and *Vitis labrusca* (cv. Bordo and Isabella) are the main varieties used to produce wine and grape juices [9]. There are many works about the biological effects of grape seed extracts obtained from *V. vinifera* [10]. However, the possibility to use *V. labrusca* varieties as a source of polyphenols is not well established.

Different methods have been developed to measure the antioxidant activity of natural compounds. This evaluation can be performed by *in vitro* or *in vivo* assays, and it is suggested to conduct both of them to get more reliable results. Among the *in vitro* assays, the ability of one compound to donate electrons to the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$) is one of the most used and reproducible assays [11].

Assays using mammalian living cells have also proven to be very useful in identifying antioxidant activity [12]. Brain cells are highly vulnerable to oxidative damage due to their high consumption of oxygen, the presence of large amounts of easily oxidizable polyunsaturated fatty acids, and an abundance of redox-active transition metal ions [13]. Lipid peroxidation in brain tissues is associated with a progressive loss of membrane permeability and cellular damage [13], leading to an increased susceptibility to various diseases, such as Parkinson's and Alzheimer's diseases [14]. Oxidative damage to lipids and proteins were evaluated in these tissues, as well as the enzymatic (catalase activity) and non-enzymatic (protein sulfhydryl assay) defenses found in mammalian cells [15].

The eukaryotic yeast *Saccharomyces cerevisiae* has been used to carry out *in vivo* assays, showing rapid and reproducible results. This yeast has been extensively studied both genetically and biochemically, and it is used for determining antioxidant activities [16,17].

The purpose of this study was to investigate the possibility to obtain flavan-3-ol compounds from winery waste seeds of *V. vinifera* (cv. Cabernet Sauvignon and Merlot) and *V. labrusca* (cv. Bordo and

Isabella) using water as a solvent and also to evaluate their antioxidant activity using *in vitro* and *in vivo* assays.

2. Experimental Section

2.1. Chemicals

Procyanidin B3, (+)-catechin, (−)-epicatechin, (−)-epigallocatechin, gallic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH•), 2,4-dinitrophenylhydrazine (DNPH), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), thiobarbituric acid (TBA) were purchased from Sigma-Aldrich (St. Louis, MO). All other reagents were of analytical grade.

2.2. Wine waste extracts

Seeds from winery wastes of *V. vinifera* (cv. Cabernet Sauvignon and Merlot) and *V. labrusca* (cv. Bordo and Isabella) were removed from the vinification tanks five days after the beginning of fermentation in January 2006. All varieties were cultivated in the northeast region of the Serra Gaucha, Rio Grande do Sul, Brazil. Voucher specimens were identified by the herbarium of the University of Caxias do Sul, Rio Grande do Sul, Brazil (*V. vinifera* HUCS32455-32456 and *V. labrusca* HUCS31065-31066). Seeds were manually separated from the rest of the winery wastes, dried in air oven at 37 °C and stored at 25 °C sheltered from light. Extracts were obtained using 5 g of seeds/100 mL of distilled water, under reflux (100 °C), for 30 minutes. After cooling to 25 °C, extracts were filtered in Millipore equipment (pore size, 0.45 μm; catalog number SFGS 047LS, Millipore Corp., São Paulo, Brazil). The extracts were freeze-dried (Edward freeze dryer) at 60 °C, 10^{-1} bar, and were stored at −20 °C. All grape seed extracts were solubilized in distilled water immediately before use.

2.3. Phenolic content of the wine waste extracts

Total phenolic content of the wine waste extracts were measured using Singleton and Rossi's (1965) modification of the Folin–Ciocalteu colorimetric method [18]. Two hundred microliters of the extracts were assayed with 1000 μL of Folin–Ciocalteu reagent and 800 μL of sodium carbonate (7.5%, w/v). The mixture was vortexed and diluted (1:10) with distilled water. After 30 minutes, the absorbance was measured at 765 nm, and the total phenolic content was expressed as mg/L catechin equivalent (CTE). Major polyphenols were assessed through chromatographic analyses carried out as described by Lamuela-Raventós and Waterhouse (1994) [19] using a HP 1100 (Palo Alto, CA) diode array UV-visible detector coupled to an HP Chem Station. A Zorbax SB C18 (250 × 4 mm), 5 μm particle size, was used for the stationary phase with a flow of 0.5 mL/min. Twenty-five microliters of extracts were injected into the HPLC system after filtration through a 0.45 μm Millipore membrane. The solvents used for the separation were as follows: solvent A (50 mM dihydrogen ammonium phosphate adjusted to pH 2.6 with orthophosphoric acid), solvent B (20% of solvent A with 80% acetonitrile) and solvent C (0.2 M orthophosphoric acid adjusted with ammonia to pH 1.5). The gradient conditions were: solvent A 100% (0–5 min), solvents A 96% and B 4% (5–15 min), solvents A 92% and B 8% (15–25 min), solvents B 8% and C 92% (25–45 min), solvents B 30% and C 70% (45–50 min),

solvents B 40% and C 60% (50–55 min), solvents B 80% and C 20% (55–60 min) and solvent A 100% (60–65 min). Chromatograms were monitored at 204 nm, and identification was based on retention times relative to authentic standards ((+)-catechin, (−)-epicatechin, (−)-epigallocatechin, procyanidin B1, B2, B3, and B4, and gallic acid). Quantification was performed using the standards by establishing calibration curves for each identified compound. Results are shown in mg/L.

2.4. In vitro *antioxidant activity*

In vitro antioxidant activity of the different wine waste extracts was measured by 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•) scavenging activity [11], catalase-like assay [15], and in brain tissue of rats. For the DPPH• assay, the extracts were added to Tris-HCl buffer (100 mM, pH 7.0) containing 250 μM DPPH• dissolved in ethanol. The tubes were kept in the dark for 20 min and absorbance was measured at 517 nm (UV-1700 spectrophotometer, Shimadzu, Kyoto, Japan). Results were calculated as IC_{50} (amount of extract necessary to scavenge 50% of DPPH• radical). Catechin was used as a control.

To assess the antioxidant activity in brain tissue, ten-day-old Wistar rats were obtained from the breeding colony of the Centro Universitário Metodista. They were maintained at approximately 25 °C, on a 12-h light/12-h dark cycle. All efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data. The experiments were performed in accordance with "Guide for the Care and Use of Laboratory Animals, DHEW, publication no. (NIH) 85-23, 1985" and approved by the local ethical committee at Universidade de Caxias do Sul. Assays were performed as described by Leipnitz *et al.* [20]. Briefly, animals were killed by decapitation without anesthesia, and the brain was rapidly excised on a Petri dish placed on ice. The cerebral cortex, cerebellum and hippocampus were dissected, weighed and kept chilled until homogenization, which was performed using a ground-glass-type Potter-Elvehjem homogenizer in 1.5% KCl. The homogenates were centrifuged (800 g) for 10 min at 4 °C, the pellets were discarded and the supernatants were used immediately. Aliquots were treated with the wine waste extracts (1.5%, v/v) for 30 min and then 5 mM hydrogen peroxide (H_2O_2) was added to the mixture. Samples were incubated for 1 h at 37 °C under constant agitation. All experiments were conducted in accordance with the Guiding Principles of the Use of Animals in Toxicology, adopted by the Society of Toxicology in July 1989.

Oxidative markers analyses included the quantification of lipid and protein damages and the activity of the antioxidant enzyme catalase. Protein sulfhydryl content was assessed as a non-enzymatic cellular defense. Lipid damages were monitored by the formation of thiobarbituric acid reactive species (TBARS) during an acid-heating reaction, which has been widely adopted as a sensitive method for measuring lipid peroxidation. First, 1000 μL of 5% trichloroacetic acid were added to 250 μL of supernatants and centrifuged at 7000 g for 10 min. Then, 1000 μL of sulfuric acid (3 M) were mixed with 1000 μL of thiobarbituric acid solution. The reaction mixture was incubated in a boiling water bath for 15 min, and cooled to room temperature. Then, 3500 μL of n-butanol were added and centrifuged at 7000 g for 5 min. The absorbance was read at 532 nm [21]. Results are expressed as nmol of TBARS/mg of protein. Oxidative damage in proteins was measured by determining the carbonyl grouping based on the reaction with dinitrophenylhydrazine (DNPH). Two hundred

microliters of DNPH (10 mM) or 200 µL of HCL (2 M) for control were added to 50 µL of supernatants. The reaction mixture was incubated in the dark for 30 minutes, with vortex every 10 minutes; after that, 250 µL of 20% trichloroacetic acid were added and centrifuged at 4000 g for 8 minutes. The supernatant was discarded and the pellet was washed 3 times with ethanol-ethyl acetate (1:1) to remove free reagent. Samples were centrifuged and pellets were redissolved in 600 µL of guanidine solution (6 M) at 37 °C for 15 minutes. Absorbance was read at 365 nm [22], and results expressed as nmol of DNPH/mg of protein. Catalase activity was determined by the hydrogen peroxide decomposition rate. Briefly, 20 µL of the wine waste extracts were added to 2910 µL phosphate buffer (pH 7.4) plus 70 µL of H_2O_2 (3 mM freshly diluted) and read on a spectrophotometer at 240 nm and values were expressed as µmol of H_2O_2/per minute per mg of protein [23]. Determination of protein sulfhydryl content was based on the reaction with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) whose absorbance was measured spectrophotometrically at 412 nm. Briefly, 0.1 mM DTNB was added to 120 µL of supernatants. This was followed by 30 min incubation at room temperature in a dark room. The sulfhydryl content is inversely correlated with oxidative damage to proteins. Results are expressed as µmol of DTNB/mg of protein [24]. Protein concentration was determined by the Bradford method [25] using bovine serum albumin as standard.

2.5. In vivo *antioxidant activity*

In vivo antioxidant activity was carried out using eukaryotic cells of the yeast *Saccharomyces cerevisiae* XV185-14C (*MATα*, *ade 2-1*, *arg 4-17*, *his 1-7*, *lys 1-1*, *trp 1-1*, *trp 5-48*, *hom 3-10*) provided by Dr. R.C. Von Borstel (Genetics Department, University of Alberta, Edmonton, AB, Canada) treated with the highest noncytotoxic concentration, 2.5% (v/v), as well as 0.5% (v/v) and 1.5% (v/v) of each extract plus H_2O_2 (4 mM). The tubes were incubated for 2 h at 28 °C. The samples were diluted in a sodium chloride solution 0.9% (p/v), seeded into a complete culture medium (10 g/L of yeast extract, 20 g/L of peptone, 20 g/L of dextrose and 20 g/L of agar-agar) and incubated for 48 h at 28 °C. After incubation, the colonies were counted, defining the total number of colonies observed on the control plate (untreated cells) as a 100% survival rate. The antioxidant activity of the extracts was evaluated by the ability of the extracts to avoid/minimize the oxidative lethal damages induced by H_2O_2, as already described [16,17].

2.6. Statistical analysis

All measurements were performed at least in triplicate, and values were averaged and reported along with the standard deviation. Data were subjected to analysis of variance (ANOVA), Tukey test, and Pearson correlation using a SPSS 12.0 software package (SPSS Inc., Chicago, IL).

3. Results and Discussion

The total phenolic content of the aqueous wine waste extracts studied in this work varied from 353.20 ± 4.60 mg/L to 751.38 ± 5.30 mg/L for the Isabella and Merlot varieties, respectively. Cabernet Sauvignon, Merlot and Bordo extracts did not show any significant differences in total phenolic content. The main phenolic compounds in all wine waste extracts were catechin and epicatechin,

followed by procyanidin B3, procyanidin B1, procyanidin B2, gallic acid, epigallocatechin, and procyanidin B4 (Table 1). This work shows that it is possible to obtain flavan-3-ol compounds from wine waste seeds using water as a solvent. This aspect is important to avoid possible organic solvent residues in the final product. It is important to mention that the aqueous extracts of *V. vinifera* studied herein present similar phenolic composition to that observed in seed extracts from Cabernet Sauvignon and Merlot varieties prepared in ethanol [26], methanol [27] and ethyl acetate/acetone [28]. Besides, we have shown here that it is possible to use *V. labrusca*, mainly Bordo variety, as source of flavan-3-ol compounds.

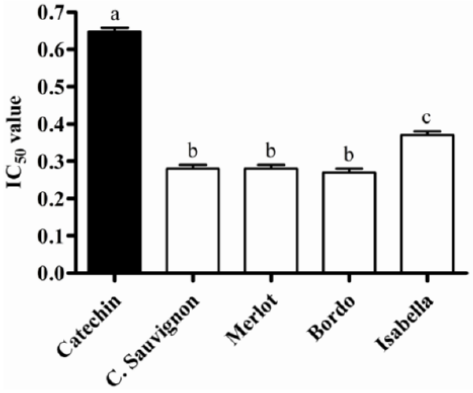
Table 1. Total polyphenol content (mg/L equivalent of catechin) and major compounds (mg/L) in the wine waste extracts.

		Major compounds							
GSE	**TPC**	**CT**	**ECT**	**EGC**	**B1**	**B2**	**B3**	**B4**	**GA**
Cabernet Sauvignon	715.59 ± 5.87 ^{a}	106.73 ± 0.34 ^{a}	71.53 ± 0.33 ^{a}	8.14 ± 1.29 ^{a}	26.54 ± 1.86 ^{ab}	15.23 ± 0.08 ^{a}	29.53 ± 2.70 ^{a}	2.89 ± 0.02 ^{a}	11.87 ± 0.17 ^{a}
Merlot	751.38 ± 5.30 ^{a}	109.57 ± 0.20 ^{a}	111.08 ± 0.05 ^{b}	7.49 ± 0.97 ^{a}	27.80 ± 0.82 ^{b}	13.73 ± 0.17 ^{a}	47.16 ± 0.45 ^{b}	2.87 ± 0.19 ^{a}	16.42 ± 1.15 ^{b}
Bordo	744.89 ± 3.13 ^{a}	169.26 ± 0.92 ^{b}	168.86 ± 2.82 ^{c}	8.96 ± 0.05 ^{a}	22.42 ± 0.51 ^{a}	19.75 ± 0.17 ^{a}	17.45 ± 0.01 ^{c}	1.85 ± 0.12 ^{ab}	12.98 ± 0.54 ^{a}
Isabella	353.20 ± 4.60 ^{b}	135.36 ± 0.99 ^{c}	112.40 ± 0.32 ^{b}	5.64 ± 0.02 ^{b}	8.86 ± 0.03 ^{c}	3.17 ± 3.64 ^{b}	9.72 ± 0.01 ^{d}	1.72 ± 0.06 ^{b}	6.88 ± 0.04 ^{c}

Results represent average values ± S.D. ^{a} Different letters indicate significant differences using analysis of variance (ANOVA) and Tukey post-hoc test ($p \leq 0.05$). TPC: total phenolic content; CT: catechin; ECT: epicatechin; EGC: epigallocatechin; B1: procyanidin B1; B2: procyanidin B2; B3: procyanidin B3; B4: procyanidin B4; GA: gallic acid.

DPPH• assay [11] was used to assess *in vitro* antioxidant activity of the wine waste extracts. All of them showed higher antioxidant activity than the standard catechin. The extracts with high levels of polyphenols content (Bordo, Cabernet Sauvignon and Merlot) also showed higher antioxidant activity (Figure 1). In fact, a strong correlation was found ($r^2 = 0.950$, $p \leq 0.01$) between the total phenolic content and the DPPH• assay, suggesting that these compounds are responsible, at least in part, for the antioxidant activity observed.

Figure 1. *In vitro* antioxidant activity of wine waste extracts. IC_{50} is the amount (%) of extracts needed to scavenge 50% of DPPH•. Catechin was used as control. The results represent mean ± S.D. of three independent experiments. Different letters are statistically different by analysis of variance (ANOVA) and Tukey's post-hoc test ($p \leq 0.05$).



Brain cells are continuously threatened by the damage caused by reactive oxygen species produced during normal oxygen metabolism or induced by exogenous sources [29]. The cerebral cortex, cerebellum and hippocampus are known to be the major integrative parts affected in various neurodegenerative disorders [30], such as amyotrophic lateral sclerosis, Alzheimer's and Parkinson's diseases [31-33]. The cerebral cortex and the hippocampus are regions associated with cognition and feedback stress control, while the cerebellum is uncharged of motor function [34]. Therefore, the biological effects of wine waste extracts in brain tissues were also studied. Results show that treatments with hydrogen peroxide induced an increase in lipid (TBARS) and protein (carbonyl) damages and in catalase activity, along with a decrease in protein sulfhydryl content in all tissues analyzed. Pre-treatment with the wine waste extracts prevented lipid and protein damages (Table 2), and the increase in catalase activity induced by H_2O_2, as well as protected the sulfhydryl content from oxidation in the cerebral cortex, cerebellum and hippocampus of rats (Table 3).

Table 2. Lipid and protein damage in the cerebral cortex, hippocampus and cerebellum of rats treated with the wine waste extracts plus hydrogen peroxide.

Treatments	Lipid damage (nmol/mg protein)			Protein damage (nmol/mg protein)		
	Cerebral Cortex	Cerebellum	Hippocampus	Cerebral Cortex	Cerebellum	Hippocampus
Control	0.89 ± 0.02 ^{a}	1.16 ± 0.17 ^{a}	1.96 ± 0.11 ^{a}	29.06 ± 3.16 ^{a}	3.45 ± 0.54 ^{a}	1.06 ± 0.30 ^{a}
H_2O_2	1.93 ± 0.03 ^{b}	1.84 ± 0.09 ^{d}	5.03 ± 0.09 ^{b}	64.37 ± 1.26 ^{b}	7.86 ± 0.27 ^{b}	3.06 ± 0.30 ^{b}
Cabernet Sauvignon + H_2O_2	0.27 ± 0.03 ^{c}	0.70 ± 0.08 ^{c}	1.89 ± 0.41 ^{ac}	10.73 ± 1.27 ^{c}	5.18 ± 0.81 ^{a}	0.85 ± 0.20 ^{a}
Merlot + H_2O_2	0.22 ± 0.03 ^{c}	0.76 ± 0.10 ^{bc}	1.36 ± 0.47 ^{ac}	11.18 ± 0.63 ^{c}	3.45 ± 0.54 ^{a}	1.07 ± 0.10 ^{a}
Bordo + H_2O_2	0.38 ± 0.05 ^{d}	0.99 ± 0.06 ^{ab}	1.37 ± 0.25 ^{c}	14.75 ± 4.42 ^{c}	4.41 ± 0.81 ^{a}	0.71 ± 0.10 ^{a}
Isabella + H_2O_2	0.46 ± 0.03 ^{d}	1.04 ± 0.13 ^{a}	2.12 ± 0.13 ^{a}	14.75 ± 0.63 ^{c}	4.60 ± 0.54 ^{a}	0.57 ± 0.10 ^{a}

Tissues were incubated for 30 min in the presence of the different extracts (1.5%) and 1 h in the presence of 5 mM H_2O_2. Data are mean ± S.D. Different letters indicate a significant difference according to analysis of variance and Tukey's post-hoc test ($p \leq 0.05$) for each tissue evaluated.

Table 3. Enzymatic and non-enzymatic defenses in cerebral cortex, hippocampus and cerebellum of rats treated with the wine waste extracts plus hydrogen peroxide.

Treatments	Catalase (μmol H_2O_2/mg protein/min)			Protein sulfhydryl content (μmol/mg protein)		
	Cerebral Cortex	Cerebellum	Hippocampus	Cerebral Cortex	Cerebellum	Hippocampus
Control	0.06 ± 0.01 ^{a}	0.18 ± 0.01 ^{a}	0.33 ± 0.01 ^{a}	27.54 ± 1.13 ^{a}	21.39 ± 1.30 ^{a}	48.91 ± 1.70 ^{a}
H_2O_2	0.09 ± 0.01 ^{b}	0.26 ± 0.01 ^{b}	0.62 ± 0.01 ^{b}	17.56 ± 1.69 ^{b}	13.95 ± 1.32 ^{b}	26.33 ± 1.77 ^{b}
Cabernet Sauvignon + H_2O_2	0.06 ± 0.01 ^{a}	0.20 ± 0.01 ^{c}	0.06 ± 0.01 ^{ac}	27.94 ± 1.69 ^{a}	33.48 ± 0.01 ^{c}	31.35 ± 1.70 ^{c}
Merlot + H_2O_2	0.02 ± 0.01 ^{d}	0.08 ± 0.01 ^{d}	0.02 ± 0.01 ^{cd}	25.94 ± 1.13 ^{a}	31.62 ± 2.63 ^{c}	36.62 ± 1.42 ^{c}
Bordo + H_2O_2	0.05 ± 0.01 ^{ac}	0.07 ± 0.01 ^{e}	0.05 ± 0.01 ^{ac}	23.15 ± 2.82 ^{ab}	37.20 ± 2.60 ^{c}	55.18 ± 3.55 ^{c}
Isabella + H_2O_2	0.03 ± 0.01 ^{cd}	0.10 ± 0.01 ^{f}	0.03 ± 0.01 ^{cd}	29.93 ± 1.13 ^{a}	38.13 ± 1.32 ^{c}	37.44 ± 0.25 ^{c}

Tissues were incubated for 30 min in the presence of the different extracts (1.5%) and 1 h in the presence of 5 mM H_2O_2. Data are mean ± S.D. Different letters indicate a significant difference according to analysis of variance and Tukey's post-hoc test ($p \leq 0.05$) for each tissue evaluated.

The TBARS assay is one of the oldest and most frequently used tests for measuring the peroxidation of fatty acids and membranes. The main product formed during lipid peroxidation is malondialdehyde, a powerful genotoxic and carcinogenic compound [14]. Proteins are also target of oxidative modification by reactive oxygen species. These reactions often lead to the modification of certain amino acid residues forming carbonyl derivates, which is linked to losses in physiological functions under pathological processes or during aging [35].

Pearson's correlations between the oxidative stress markers in the cerebral cortex, cerebellum and hippocampus tissues and the phenolic content levels of the different extracts are shown in Table 4. In a general way, it is possible to observe that polyphenols present negative correlations with lipid and protein oxidative damages, suggesting that these compounds are able to prevent the damages induced by H_2O_2.

Table 4. Pearson correlations and their statistical significance among the wine waste extract constituents and oxidative parameters evaluated.

	TPC	CT	ECT	EGC	B1	B2	B3	B4	GA
Cerebral cortex lipid damage	−0.752 **	−0.686 *	−0.616 *	−0.755 **	−0.745 **	−0.586 *	−0.719 **	−0.833 **	−0.759 **
Cerebellum lipid damage	−0.794 **	−0.650 *	−0.587 *	−0.765 **	−0.801 **	−0.682 *	−0.744 **	−0.840 **	−0.780 **
Hippocampus lipid damage	−0.718 **	−0.608 *	−0.634 *	−0.665 *	−0.706 *	−0.617 *	−0.725 **	−0.696 **	−0.769 **
Cerebral cortex protein damage	−0.781 **	−0.771 **	−0.714 **	−0.803 **	−0.746 **	−0.658 *	−0.654 *	−0.804 **	−0.769 **
Hippocampus protein damage	−0.580 *	−0.694 *	−0.640 *	−0.656 *	n.f.	n.f.	n.f.	−0.586 *	n.f.

TPC: total phenolic content; CT: catechin; ECT: epicatechin; EGC: epigallocatechin; B1: procyanidin B1; B2: procyanidin B2; B3: procyanidin B3; B4: procyanidin B4; GA: gallic acid; n.f.: not found. * Significant Pearson correlation for $p \leq 0.05$ and ** for $p \leq 0.01$.

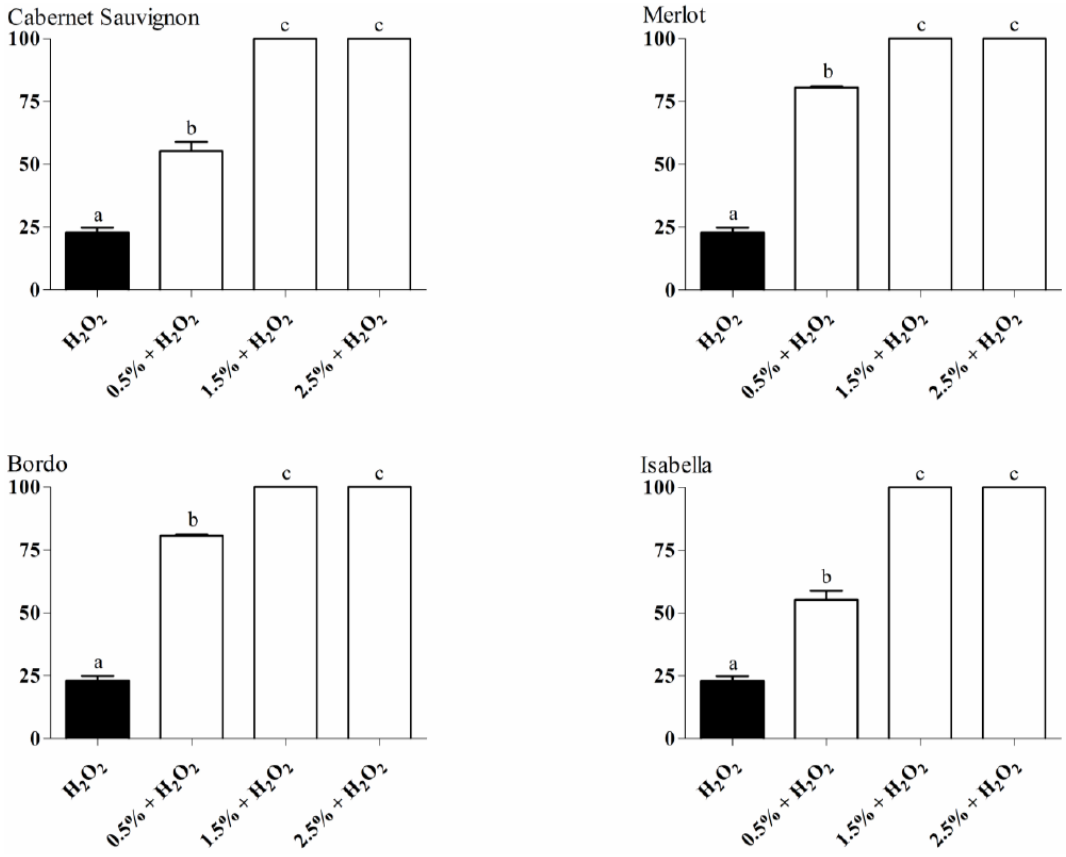
To assess the *in vivo* antioxidant activity of wine waste extracts, eukaryotic cells of *S. cerevisiae* treated with noncytotoxic concentrations of the extracts plus H_2O_2 were used. Results (Figure 2) show that all extracts were able to protect the yeast cells against the damage induced by H_2O_2. At higher concentrations (1.5 and 2.5%) of the extracts, the wine waste extracts completely prevented (100% survival) the cytotoxic effects of H_2O_2. The following positive correlations between *in vivo* antioxidant activity and total phenolic content for each extract were found: Bordo ($r^2 = 0.877$, $p \leq 0.05$), Isabella ($r^2 = 0.847$, $p \leq 0.05$), Cabernet Sauvignon ($r^2 = 0.867$, $p \leq 0.05$) and Merlot ($r^2 = 0.935$, $p \leq 0.05$), suggesting the role of these compounds in the *in vivo* antioxidant activity observed in this work.

The antioxidant mechanisms of the phenolic compounds are complex and are still being studied. In general, they can prevent the formation of reactive species by chelating trace elements involved in free radical production, scavenge reactive species, and upregulate or protect antioxidant defenses [14].

Apart from known vitamins and minerals, phenolic compounds may be one of the most widely marketed groups of dietary supplements. This class of plant metabolites shows antibacterial effects [36],

an ability to reduce blood pressure [37] and antioxidant, anti-inflammatory, antimutagenic and/or anticarcinogenic effects, at least in *in vitro* systems [38-40].

Figure 2. Survival of *S. cerevisiae* cells treated with wine waste extracts plus hydrogen peroxide. Data are mean ± S.D. Different letters indicate significant differences using analysis of variance (ANOVA) and Tukey's post-hoc test ($p \leq 0.05$).



Supplementation of bioavailable and safe natural products, as polyphenols, is important to complement diets poor in antioxidants that we consume daily. Although further studies are necessary, the flavan-3-ol compounds studied herein show potential to be used as antioxidants. Studies about the safety of these extracts are being conducted, but it is already known that procyanidin extracts from grape seeds, assessed in compliance with the U.S. Environmental Protection Agency's Health Effects Test Guidelines and the Toxic Substances Control Act, have shown to be safe for human intake [41].

4. Conclusions

The data presented herein has shown that it is possible to obtain flavan-3-ol compounds from wine wastes using water as a solvent. As we hypothesized, both *V. vinifera* and *V. labrusca* species can be used to obtain extracts with important *in vitro* and *in vivo* antioxidant activity, which could be used as dietary supplements.

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Declare

Each of the authors declares no conflict of interest.

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8.2 Anexo 2

Antioxidant and anti-inflammatory activities of winery wastes seeds of Vitis labrusca

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Antioxidant and anti-inflammatory activities of winery wastes seeds of *Vitis labrusca*

Atividade antioxidante e anti-inflamatória de sementes de resíduos de vinificação de *Vitis labrusca*

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ABSTRACT

*There are many studies about the biological activities of **Vitis vinifera** grape seeds, which are rich in phenolic compounds, known by their several health beneficial effects. However, until now there is no data about biological activities of the seeds of **V. labrusca**, specie found in South and North America. Every year, the global wine production (around 260 million hL) generates about 19.5 million ton of wastes, which are usually discarded in the environment. The aim of this research was to evaluate the antioxidant and anti-inflammatory activities of aqueous extracts of seeds from wine wastes of **Vitis labrusca** (cv. 'Bordo' and 'Isabella'). Both extracts showed significant antioxidant and anti-inflammatory activities, which are positively correlated with total phenolic content, suggesting that these compounds might be the major contributors to the biological activity of these extracts. These results indicate that water extraction from winery wastes is an option to obtain phenolic compounds with antioxidant and anti-inflammatory activities helping to maintain environmental balance.*

Key words: *V. labrusca, winery wastes, antioxidant, anti-inflammatory.*

RESUMO

*Apesar de existirem vários estudos sobre a atividade biológica de sementes de uva de **Vitis vinifera**, ricas em compostos fenólicos com reconhecidos efeitos benéficos à saúde, não existem, até o momento, dados a respeito da atividade biológica de sementes de **V. labrusca**, espécie amplamente encontrada na América do Sul e do Norte. A cada ano, a produção mundial de vinho (cerca de 260 milhões de hL) gera, aproximadamente, 19,5 milhões de toneladas de resíduos, usualmente descartados no meio ambiente. Em vista disso, o objetivo deste estudo foi avaliar as atividades antioxidante e anti-inflamatória de extratos aquosos de sementes de resíduos de vinificação de **V. labrusca** (cv. 'Bordo' e 'Isabel'). Os resultados mostraram que ambos os extratos apresentam significante atividade antioxidante e anti-inflamatória, as quais apresentam correlação positiva com o conteúdo de compostos fenólicos dos extratos, sugerindo que estes podem contribuir, significativamente, para a atividade biológica observada. Estes resultados mostram que é possível obter compostos fenólicos com atividades antioxidante e anti-inflamatória utilizando extração aquosa, além de contribuir com o equilíbrio do meio ambiente.*

Palavras-chave: *V. labrusca, resíduos de vinificação, antioxidante, anti-inflamatório.*

INTRODUCTION

There are several studies about the biological activities of ***Vitis vinifera*** grape seed extracts (for review see XIA et al., 2010). These studies using organic solvents (TORRES et al., 2002; XIA et al., 2010) have limited use due to the high cost of the extraction processes. The extraction of phenolic compounds with non-organic solvents is of interest mainly to the pharmaceutical industries, as they are able to minimize pathologies associated with oxidative stress, such as atherosclerosis, diabetes, cancer, inflammatory and neurological diseases (XIA et al., 2010).

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There are few studies about the biological activities of *V. labrusca* specie (RIZZON et al., 2000, VEDANA et al., 2008, DANI et al., 2010), however, until now there are no data about the potential of using *V. labrusca* seeds as a source of biologically active compounds. *V. labrusca* (mainly the Bordo and Isabella varieties) is the main grape species found in South and North America, and it is widely used to produce wines and grape juices (SOARES DE MOURA et al., 2002; POLLEFEYS & BOUSQUET, 2003).

Every year, the global wine production (around 260 million hL) generates about 19.5 million ton of wastes (OIV, 2010), which are generally used as fertilizer or simply discarded in the environment (TORRES et al., 2002). Although some polyphenols are transferred from the grapes to the wine during vinification, and there is a potential loss of some of these compounds by oxidation during the industrial process, the seed wastes are still good sources of phenolic compounds (TORRES et al., 2002).

This research aimed to assess the antioxidant and anti-inflammatory activities of aqueous extract of *V. labrusca* winery wastes seeds (Bordo and Isabella varieties).

MATERIALS AND METHODS

Winery wastes of *V. labrusca* (cv. 'Bordo' and 'Isabella') were used in this study. Both varieties were cultivated in the northeastern region of the Serra Gaucha, Rio Grande do Sul, Brazil. Voucher specimens (HUCS31065-31066) were identified by the herbarium of the University of Caxias do Sul, Rio Grande do Sul, Brazil. Seeds were removed from vinification tanks in January 2006, five days after fermentation beginning. They were immediately separated from the remainder of the winery wastes manually, dried in an air oven at 37°C and sheltered from light. Grape seeds were pounded in a knife mill (Quimis, Brazil) and the extracts were prepared with 5g seeds 100mL^{-1} distilled water under reflux (100°C) for 30 minutes. Extracts were cooled to 25°C, filtered in (pore size, 0.45µm, Millipore Corp., Sao Paulo, Brazil) and freeze-dried at -60°C, 10^{-1} bar. Total phenolic content and the major constituents of these extracts were described in SCOLA et al. (2010) and are shown in table 1. No alkaloids, saponins or terpenoids were found in the extracts.

The antioxidant activity of the *V. labrusca* extracts was assayed by total reactive antioxidant potential (TRAP) (DRESCH et al., 2009), total antioxidant reactivity (TAR) (LISSI et al., 1995), and thiobarbituric acid reactive species (TBARS) (SILVA et al., 2007) assays. TRAP and TAR assays were used to determine the capacity of extracts to trap a flow of water-soluble peroxyl radicals produced at constant rate, through thermal decomposition of AAPH, as previously described. Briefly, the reaction mixture (4mL), containing AAPH (10mM) and luminol (4mM) in glycine buffer (0.1M), pH 8.6, was incubated at 21°C for 2h. AAPH is a source of peroxyl radicals that react with luminol yielding chemiluminescence (CL). The system was calibrated using trolox. The addition of 10µL of the extracts or trolox decreases the CL proportionally to its antioxidant potential. The TRAP profile was obtained by measuring the CL emission in a liquid scintillation counter (Wallac 1409) as counts per minute (CPM). CL intensity was monitored for 50 min after adding the extracts (2.5µg mL^{-1}) or trolox (200nM). Results were calculated as area under curve (AUC) of the CL profile and were expressed as percent of inhibition. TAR index was determined by measuring the initial decrease of luminol luminescence calculated as Io/I ratio, where Io is the initial emission of CL (before adding extracts or trolox) and I is the instantaneous CL intensity after adding an aliquot of the sample or the reference compound (trolox).

TBARS were assayed to measure the antioxidant potential of *V. labrusca* extracts against a lipid peroxidation cascade (including different reactive oxygen species, such as peroxyl radicals, superoxide, hydrogen peroxide, and hydroxyl) generated from egg yolk lipid homogenate. Briefly, fresh egg yolk was

Table 1 - Total polyphenol content (mg L^{-1} of catechin equivalent) and major compounds (mg/L) in *V. labrusca* grape seed extracts

		Major compounds							
Extracts	TPC (mg L^{-1} CAE) extract	Catechin	Epicatechin	Epigallocatechin	Procyanidin B1	Procyanidin B2	Procyanidin B3	Procyanidin B4	Gallic acid
Bordo	744.9±3.1^{a}	169.3±0.9^{a}	168.9±2.8^{a}	8.9±0.1^{a}	22.4±0.5^{a}	19.7±0.2^{a}	17.4±0.1^{a}	1.8±0.1^{a}	12.9±0.6^{a}
Isabella	353.2±4.6^{b}	135.4±0.9^{b}	112.4±0.3^{b}	5.6±0.1^{b}	8.9±0.1^{b}	3.2±0.7^{b}	9.7±0.1^{b}	1.7±0.1^{a}	6.9±0.1^{b}

TPC, total phenolic content; CAE, catechin equivalents. *Different letters indicate significant differences (P=0.05). Total phenolic content was measured using Folin-Ciocalteau colorimetric method and major compounds were measured by HPLC. These results are adapted from SCOLA et al. (2010).

homogenized (1% w/v) in 20mM phosphate buffer (pH 7.4), 1mL of homogenate was sonicated (10s at potency 4) and then homogenized with 0.1 mL of extracts (2.5µg mL^{-1}) or positive controls were prepared immediately before use. Lipid peroxidation was induced by adding 0.1mL of AAPH solution (0.12M). AAPH was used as positive control. Reactions were carried out for 30min at 37°C. After cooling, samples (0.5mL) were centrifuged with 0.5mL of trichloroacetic acid (15%) at 1200g for 10min. A 0.5mL aliquot of supernatant was mixed with 0.5mL of TBA (0.67%) and heated at 95°C for 30 min. After cooling, sample absorbance was measured using a spectrophotometer at 532nm. Results were expressed as % of TBARS in relation to the positive control.

The anti-inflammatory activity was assessed in three-months-old Wistar rats (250-350g) from our breeding colony. They were caged in groups of five with free access to food and water and were maintained on a 12-h light-dark cycle (7-19h) at 23±1°C. All experimental procedures were performed in accordance with the National Institute of Health Guide for Care and Use of Laboratory Animals (NIH publication, revised 1985) and were carried out according to the regulations of the Brazilian College of Animal Experimentation, COBEA. All efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data. Rats (n=6) were treated intraperitoneally with saline or 10mg kg^{-1} body wt of ***V. labrusca*** extracts 30 minutes before induction of peritonitis through injection of 0.2mL of carrageenan 1% (PETRONILHO et al., 2010). Four hours after inducing inflammation, animals were euthanized, and pleural exudates from each animal were harvested by washing the pleural cavity with 2mL of sterile saline solution for measuring total and differential cell count, lactate dehydrogenase activity (LDH), TNF-alpha levels and total proteins. Total cells in the pleural exudate were enumerated in a Neubauer chamber to obtain total leukocyte counts. LDH and TNF-alpha levels were determined with commercially kits (Labtest Diagnóstica, Brazil and Calbiochem-Novabiochem Corporation, USA, respectivelly). Total protein was measured by Lowry method using bovine serum albumin as the standard (LOWRY et al., 1951).

Thiobarbituric acid (TBA), luminol (3-aminophthalhydrazide), carrageenan and formalin were purchased from Sigma-Aldrich (St. Louis, MO). 2,2'-azobis(2-methylpropionamidine) dihydrochloride (AAPH) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox) were purchased from Aldrich Chemical (Milwaukee, WI). Acetic acid and glycine were purchased from Nuclear (Diadema, SP, Brazil). Trichloroacetic acid (TCA) and sodium carbonate were purchased from Synth (Diadema, SP, Brazil). (+)-Catechin, (-)-epicatechin, (-)-epigallocatechin, procyanidins B1, B2, B3 and B4 and gallic acid were purchased from Sigma-Aldrich (St. Louis, MO). All other reagents were of analytical grade.

TBARS, TRAP and TAR measurements were performed through four independent tests (in triplicate for each one). Values were averaged and expressed along with the standard deviation. For the anti-inflammatory activity, the means and the standard deviation of data obtained from 6 rats per group were used. Results were subjected to analysis of variance (ANOVA), Tukey´s post-hoc test and Pearson´s correlation using a SPSS 12.0 software package (SPSS Inc., Chicago, IL).

RESULTS

Results show that both ***V. labrusca*** extracts (Bordo and Isabella) have the ability to reduce the luminol-enhanced chemiluminescence, indicating the presence of compounds with peroxyl scavenging properties higher than the trolox activity (Figure 1A). TAR-index results (Figure 1B) show that both extracts are able to scavenge peroxyl radicals, diminishing CL intensity after the addition of the extracts in comparison with trolox. The Bordo extract shows higher antioxidant activity against lipid oxidative damage than the Isabella extract (Figure 1C). In fact, a positive correlation between total phenolic content and TAR index (r^2=0.920, P≤ 0.01) was found. Interestingly, no correlations were found among specific phenolic compounds and antioxidant activity assessed by TRAP/TAR assays. On the other hand, negative correlations between TBARS levels and specific polyphenols were found, as follow: catechin (r^2=-0.998, P≤ 0.05), procyanidin B1 (r^2=-0.999, P≤ 0.01), procyanidin B2 (r^2=-0.997, P≤0.01) and epicatechin (r2=-0.999, P≤0.01).

The intraperitoneal injection of 0.2mL of 1 % carrageenan into the pleural cavity of rats induced an inflammatory reaction characterized by exudate formation and cell migration, when compared to the control group (saline, Figure 2). Both the Bordo and the Isabella extracts show no significant decrease in the total cell number (Figure 2A) or polymorphonuclear migration (Figure 2B). However, an important decrease in lymphocyte migration to the inflammatory site (Figure 2C) was observed. Treatments with both extracts showed no effects on TNF-alpha levels, LDH, or total proteins in the exudate (data not shown). Positive correlations between the diminished

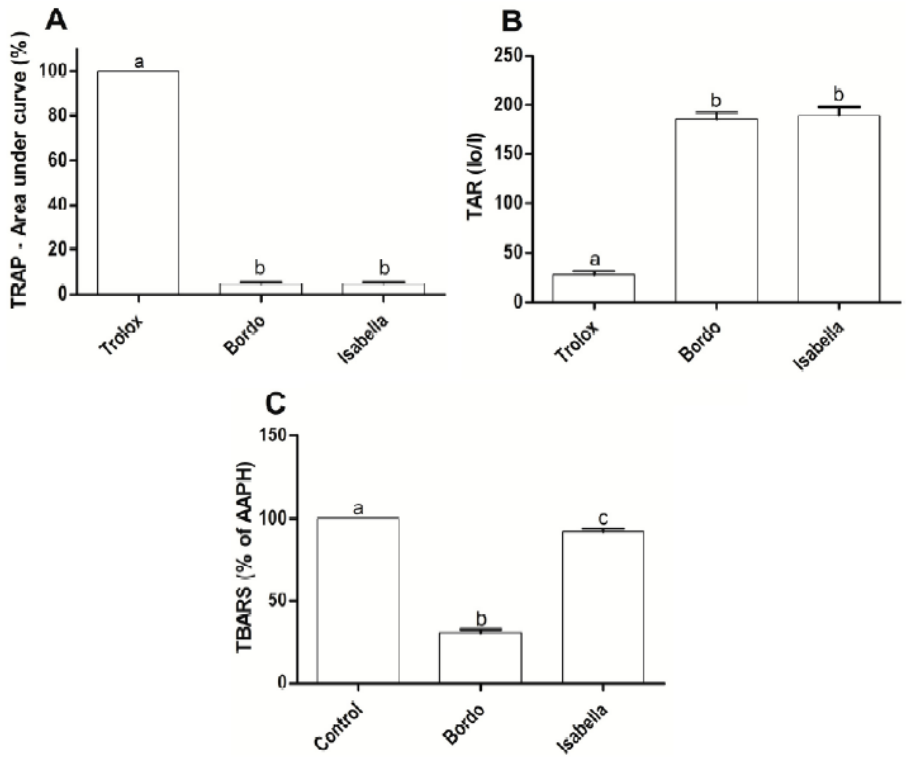

Figure 1 - Antioxidant activity of *V. labrusca* extracts. A) Total reactive antioxidant potential (TRAP) of *V. labrusca* extracts (2.5µg mL^{-1}). Bars represent percent of inhibition relative to the system area generated during luminescence reading of AAPH alone. B) The total antioxidant reactivity was calculated as the ratio of light intensity in the absence of samples and the light intensity right after extracts (2.5µg mL^{-1}) addition and expressed as percent of inhibition (I_o/I) using the same raw data. C) TBARS *in vitro*: lipid extracted from egg yolk was subjected to oxidative damage by incubation with AAPH, and the ability of the extracts (2.5µg mL^{-1}) to prevent TBARS formation was analyzed. Data is mean ± S.D. of three independent tests. Different letters indicate significant difference using analysis of variance (ANOVA) and Tukey's post-hoc test ($P \leq 0.01$).

lymphocyte migration levels and specific polyphenols were found: procyanidin B1 (r^2=0.999, $P \leq 0.01$), procyanidin B2 (r^2=0.976, $P \leq 0.05$), epigallocatechin (r^2=0.900, $P \leq 0.01$), and epicatechin (r^2=0.998, $P \leq 0.01$).

DISCUSSION

Both *V. labrusca* extracts (Bordo and Isabella) show antioxidant activity assessed by TRAP/TAR (Figure 1A and 1B) and TBARS (Figure 1C) assays. The Bordo extract showed higher potential to avoid oxidative damage to lipids, measured by TBARS (Figure 1C), and higher polyphenol content than the Isabella extract (Table 1). Several studies demonstrate that *V. vinifera* varieties show important antioxidant activities (for review see XIA et al., 2010). On the other hand, there is only one research about the antioxidant activity of *V. labrusca* leaves (DANI et al., 2010). This is the first research that shows biological activities for *V. labrusca* seeds from winery wastes. It is possible that phenolic compounds might be the major contributors to the biological activities of *V. labrusca* extracts related in this research. The antioxidative mechanism of phenolic compounds is mainly ascribed to their free radical-scavenging and metal-chelating properties, as well as their effects on cell-signaling pathways and on gene expression (SOOBRATTEE et al., 2005).

Carrageenan is a high-molecular-weight sulfated polysaccharide, which is widely used in pharmacology to induce local inflammation (paw edema and pleurisy) in rats. Carrageenan-induced pleurisy is a well-characterized experimental model of inflammation, which permits the quantification of exudates and cellular migration (PETRONILHO et al., 2010). The administration of carrageenan into the pleural space leads to pleurisy, characterized by an immediate

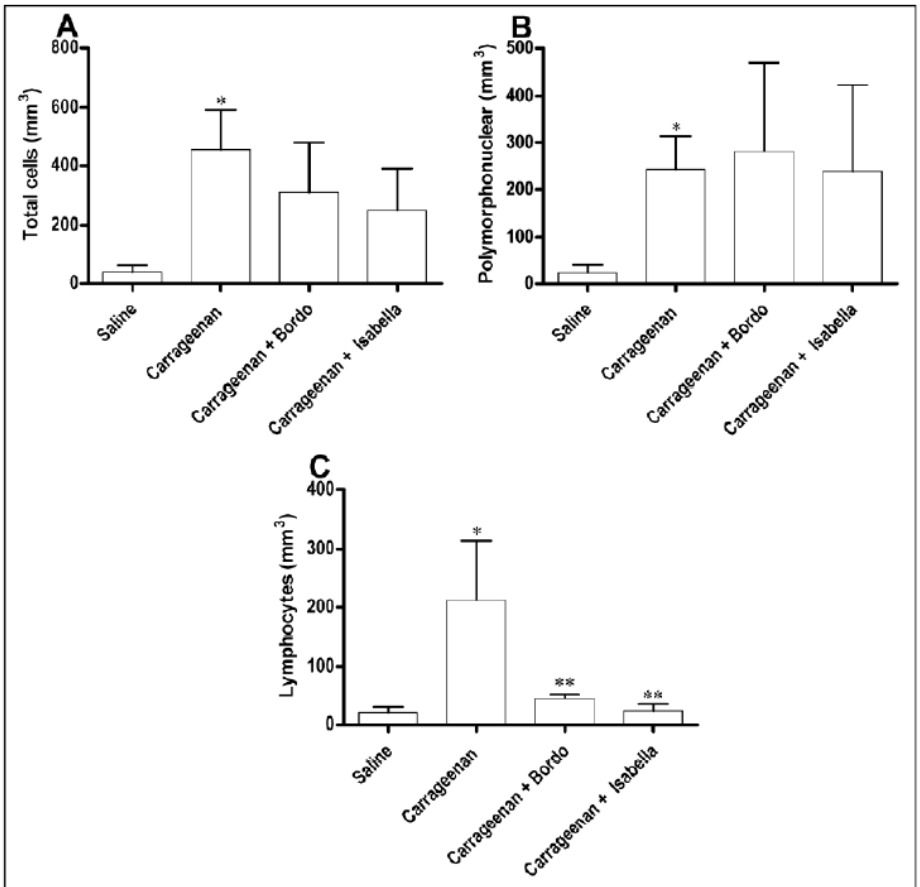

Figure 2 - Effect of the different *V. labrusca* extracts on leukocyte migration into the peritoneal cavity induced by carrageenan in rats. Rats were pre-treated with saline or extracts (10mg kg^{-1} body wt., i.p.) 30 minutes before carrageenan (2mL of 1% i.p.) induced peritonitis. Cell counts were performed 4h after carrageenan injection. Total cells migration (A), polymorphonuclear migration (B) and lymphocytes migration (C) into pleural cavity of rats. Data is mean ± S.D. * Significant differences from saline ($P \leq 0.05$). ** Significant differences from carrageenan ($P \leq 0.05$).

polymorphonuclear infiltration. Besides infiltration, pleurisy induced by carrageenan is characterized by the production of neutrophil-derived reactive oxygen species, such as hydrogen peroxide (H_2O_2), superoxide anion and hydroxyl radical, and neutrophil-derived mediators such as TNF-alpha (SALVEMINI et al., 1996). Evidence from the literature shows that the production of reactive oxygen and nitrogen species occurs at the site of inflammation and contributes to tissue damage (SALVEMINI et al., 1996).

Both Bordo and Isabella extracts presented a significant decrease of lymphocyte migration to the inflammation site (Figure 2C). These data suggest the participation of these compounds in the biological effect observed. Polyphenols are powerful antioxidants and exert anti-inflammatory activities in rats, mice and humans (XIA et al., 2010). Extracts from grape skins and seeds of *V. rotundifolia* inhibited mouse ear inflammation, edema, and polymorphonuclear leukocyte infiltration induced by 12-O-tetradecanoylphorbol 13-acetate (BRALLEY et al., 2007).

CONCLUSION

These data shows that it is possible to obtain aqueous extracts from winery wastes of *V. labrusca* with important antioxidant and anti-inflammatory activities. Besides these biological effects, the use of these wastes could help to maintain environmental balance.

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8.3 Anexo 3

A Fresh Look at Complex I in Microarray Data: Clues to Understanding Disease-Specific Mitochondrial Alterations in Bipolar Disorder

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CORRESPONDENCE

A Fresh Look at Complex I in Microarray Data: Clues to Understanding Disease-Specific Mitochondrial Alterations in Bipolar Disorder

To the Editor:

Mitochondrial dysfunction and the consequent generation of oxidative stress damage have consistently been reported in bipolar disorder (BD) and schizophrenia (SCZ). Microarray studies, in which the expression of many electron transport chain (ETC) genes from complexes I through V have been found to be decreased in patients with BD in the frontal cortex (1–5) and hippocampus (6), are a major source of evidence for this mechanism. Mitochondrial ETC complex I is one of the major sites for the generation of reactive oxygen species (ROS) (7). Recently, *Biological Psychiatry* (2012; volume 71, issue 11) addressed the implications of using antioxidants for the maintenance of redox balance in psychiatric disorders (8–10). Considering the complexity of mitochondrial complex I and its significance for psychiatric disorders, we re-examined the reported microarray findings and grouped the altered complex I subunits by their relevance for ROS generation.

Complex I is a large complex consisting of 45 or 46 subunits, each of which plays a unique role for the structure or activity of this complex, or ROS generation (11,12). Subunits of complex I are arranged in four main subcomplexes, λ, α-λ, γ, and β. The hydrophilic arm, which is responsible for the transfer of electrons, contains subcomplexes λ and α-λ, and the hydrophobic arm, which is largely responsible for the pumping of protons, consists of γ and β. Upon reviewing the literature, we identified 34 microarray studies examining BD, SCZ, or both. Of these studies, we have selected the microarray studies that included probes for complex I genes. Ultimately, 10 studies were selected to be included in this report because they reported alterations in complex I genes in BD or SCZ. Microarrays enable the examination of the expression pattern of a significant portion of the human genome encompassing multiple systems, making it ideal for the exploration of the pathophysiology of complex disorders such as BD and SCZ (13). Eighteen genes were found to be reduced or increased in their levels of expression in BD or SCZ, of which 8 were found to be altered in BD, 6 in SCZ, and, surprisingly, only 4 genes were found to be altered in both BD and SCZ (Figure 1). Combined findings of these studies revealed that in BD, expression of iron-sulfur cluster-containing subunits within the hydrophilic arm were reduced, suggesting that patients with BD may be more prone to having a dysfunction in the electron transfer process. For instance, *NDUFV1*, which contains FMN and N3, and initiates the electron transfer process from nicotinamide adenine dinucleotide to the iron-sulfur clusters, was found to be downregulated in BD. Also, *NDUFS1*, which contains N1b, N4, N5, and N7, and NDUFS8, which contains N6a and N6b, were also found to be downregulated. Finally, *NDUFS7*, which contains N2 and is responsible for the reduction of ubiquinone to ubiquinol, was also found to be reduced in BD (14) (Figure 1). Although four subunits were found to be downregulated in the α-λ subcomplex in BD, these subunits are noncatalytic, and we therefore focus on the λ subunit. In contrast, gene expression alterations in SCZ were found to be scattered throughout complex I, with up- and downregulation, and did not include alterations in subunits directly involved in electron transfer (15–19). These data may suggest that although patients with both BD and SCZ may have a reduction in complex I functionality, patients with BD may be more prone to deficiencies in the electron transfer process, which could increase the probability of electrons escaping the electron transport chain to react with molecular oxygen, causing a cascade of reactions to increase the production of ROS such as the hydroxyl radical (7). In fact, gene expression levels of *NDUSF7* was found to be reduced in two microarray studies in BD (1,20), and this finding was supported by a separate study in which protein levels of *NDUFS7* were found to be reduced in patients with BD (21). Importantly, this reduction in *NDUFS7* levels was found to be correlated with decreased complex I activity (21). This indicates that altered gene expression identified in microarray studies could have functional implications for complex I activity and ROS generation.

There are several caveats to these data. For example, different platforms were used for data acquisition and analysis (6,13), which could generate variability between studies. Although postmortem brain allows for direct investigation of human pathology, there are

Disease	Area	Iλ	Iα-λ	Iγ	Iβ	References
BD	BA10/46 PFC		NDUFA8↓		NDUFB5↓	(2)
BD	HYP	NDUFV1↓ NDUFS1↓ NDUFS2↓ NDUFS3↓	NDUFAB1↓ NDUFA1↓ NDUFA2↓		NDUFB5↓	(6)
BD	BA46	NDUFS3↓ NDUFS7↓	NDUFAB1↓		NDUFB2↓	(20)
BD	BA9/46	NDUFS7↓ NDUFS8↓				(1)
BD	BA46	NDUFS8↓				(3)
SCZ	BA46 DLPFC	NDUFA5↑				(15)
SCZ	BA9/46 DLPFC	NDUFA4↑				(16)
SCZ	BA9 PFC		NDUFA9↓		NDUFB2↓ NDUFB3↓	(17)
SCZ	DLPFC	NDUFA2↑		NDUFC1↑	NDUFB5↑	(18)
SCZ	BA46	NDUFS2↓			NDUFB2↓	(20)
SCZ	STG		NDUFA6↓			(19)

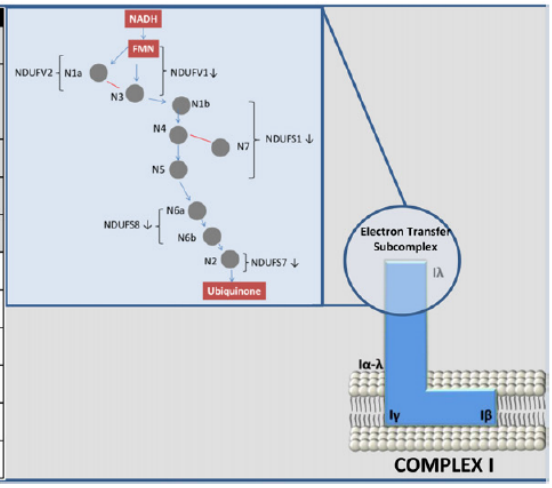

Figure 1. Summary of mitochondrial complex I gene alterations in bipolar disorder (BD) and schizophrenia (SCZ). Gene alterations and direction of alteration (arrows) in BD (highlighted in gray) and SCZ are divided into brain areas and subcomplexes where the alterations were found. Downregulations are shown in blue, and upregulations are shown in red. Mitochondrial complex I subunits *NDUFV1*, *NDUFS1*, *NDUFS8*, and *NDUFS7* within the λ subcomplex were found to be downregulated in BD. These subunits contain iron-sulfur clusters that are directly involved in the electron transfer process from NADH to ubiquinone. BA, Brodmann area; DLPFC, dorsolateral prefrontal cortex; FMN, flavin mononucleotide; HYP, hippocampus; NADH, nicotinamide adenine dinucleotide; PFC, prefrontal cortex; STG, superior temporal gyrus.

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inherent limitations involved in the use of samples acquired postmortem, such as pH, postmortem interval, and small sample size (20,21).

In summary, the data from the microarray studies discussed here suggest important differences in the expression of complex I genes between BD and SCZ. In patients with BD, there is downregulation specifically in genes involved in electron transfer in complex I. On the other hand, altered genes in SCZ were found to be scattered through complex I and include increased as well as decreased expression levels. The findings reported here suggest that in bipolar disorder, emphasis must be placed on the electron transfer chain, which may contribute to the elucidation of the pathogenesis of this condition.

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